UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Ecología

TESIS DOCTORAL

Estrategias de utilización del agua por las plantas en pinares naturales y de repoblación del sureste ibérico semiárido

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Cristina Moreno Gutiérrez

Directores

José Ignacio Querejeta
Paolo Cherubini

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Bajo la dirección de los Doctores

José Ignacio Querejeta Mercader y Paolo Cherubini

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CSIC
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A mi familia, en especial a César

“El resultado más importante
de un estudio racional de la naturaleza
es recoger la unidad y la armonía en esta
inmensa acumulación de cosas y de fuerzas”

Alexander Von Humboldt

"*No hay inversión más rentable*
que la del conocimiento"

Benjamin Franklin

ÍNDICE

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ABSTRACT

Drylands cover around 41% of Earth's land surface and include 20% of the major centers of global plant diversity. Drylands are especially vulnerable to climate change and desertification. Maintenance of biodiversity in drylands is crucial to prevent those negative impacts. In order to do so, it is important to understand how dryland plant communities are organized and the processes that allow plant species coexistence in these severely water limited environments. Afforested plantations of *Pinus halepensis* Mill., that were extensively planted in semiarid areas of the Mediterranean basin during the second half of the XXth century are particularly vulnerable to climate change and drought intensification.

The aim of the present PhD thesis was to investigate the water use strategies of some of the most common plant species naturally occurring in the semiarid Iberian SE, and to assess how plant-plant interactions influence the resource acquisition and physiological processes of vegetation in natural and afforested *P. halepensis* woodlands. We also wanted to assess how thinning, one of the most common silvicultural practices, influences the physiology of the remaining trees. Given the potential of isotopic techniques in ecophysiological studies, we wanted to evaluate their utility for investigating plant water use strategies and plant-plant interactions involving water in a semiarid ecosystem of the Iberian SE.

We measured the carbon, oxygen and nitrogen isotopic composition of leaves ($\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$, respectively) and the $\delta^{18}O$ of stem water in ten target plant species representing diverse taxonomic groups and life-forms. We applied dendroecological techniques and measured $\delta^{13}C$ and $\delta^{18}O$ in cellulose extracted from growth rings of *P. halepensis* and *Rhamnus lycioides* L. In addition to conducting isotopic analyses, we also measured plant growth, foliar nutrient concentrations, stem water potentials and leaf gas exchange.

Within and across plant species, leaf $\delta^{18}O$ and $\delta^{13}C$ were reliable indicators of stomatal conductance (g_s) and intrinsic water use efficiency (WUE_i; the ratio between photosynthetic rate and g_s), respectively. Across species, variations in leaf $\delta^{18}O$ and $\delta^{13}C$ revealed a continuous ecophysiological gradient of plant water use strategies ranging from "profligate/opportunistic" (high g_s, low WUE_i) to "conservative" (low g_s, high WUE_i). Broad inter-specific variation in $\delta^{18}O$ and $\delta^{13}C$ was largely determined by

differences in g_s, which were associated to differences in the soil depth from which plants extract water. Contrasting water use patterns among coexisting plant species may allow the temporal and spatial partitioning of the resource that is most limiting to vegetation (i.e. soil water) and thus, may foster plant species coexistence and ecosystem primary productivity in semiarid Mediterranean habitats.

However, competition for soil resources among neighbouring plants is key process structuring plant communities in semiarid regions, and indeed we found evidence of intense intra-specific competition for water among *P. halepensis* trees growing in afforested plantations, as well as inter-specific competition for water and nutrients between *P. halepensis* and *R. lycioides*.

We conducted a retrospective comparison (from 1967 to 2007) of the long-term physiological performance of *P. halepensis* trees between two types of stands with sharply contrasting structure and density (dense afforested plantations vs. neighbouring open woodlands with widely scattered pine trees). We found that tree ring width and isotope composition ($\delta^{13}C$ and $\delta^{18}O$) were strongly correlated with rainfall amount (and thus, with soil water availability) at interannual scales. Trees in dense afforested stands showed consistently higher $\delta^{18}O$ values in latewood cellulose (but similar $\delta^{13}C$) than those in neighbouring open woodlands, thus indicating lower stomatal conductance and photosynthesis rates in the former, but little difference in water use efficiency between stand types. Trees in dense afforested stands were more water stressed and showed lower radial growth due to strong intra-specific competition for water.

Due to intense intra-specific competition for water in *P. halepensis* plantations, trees responded quickly to silvicultural thinning activities in the afforested stands. Remaining trees in heavily thinned (low-density) stands showed lower leaf $\delta^{18}O$, and higher stomatal conductance, photosynthetic rate and radial growth than those in moderately thinned (medium density) stands. Higher photosynthetic rate in the remaining trees of heavily thinned stands was largely attributable to reduced stomatal limitation to CO_2 uptake. Leaf $\delta^{13}C$ and intrinsic water use efficiency were unaffected by stand density manipulation because stomatal conductance and photosynthetic rate increased roughly in parallel after thinning, thus illustrating the utility of the dual isotope approach in these situations. Simultaneous measurement of $\delta^{18}O$ and $\delta^{13}C$ in bulk leaf material helped to characterize the leaf-level physiological response of the

remaining trees to different thinning intensities at an early stage (≈ 12 months after thinning).

We also assessed the outcome of plant-plant interactions between *P. halepensis* and *R. lycioides*. In order to do so, we performed a retrospective comparison (1989-2007) of growth ring widths, $\delta^{13}C$ and $\delta^{18}O$ between *R. lycioides* shrubs growing within *P. halepensis* plantations vs. shrubs growing in neighbouring open woodlands. We also measured leaf $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$ and foliar nutrient concentrations in *R. lycioides* shrubs growing at different distances from the nearest *P. halepensis* tree in dense afforested plantations. Results suggested strong inter-specific competition for water and nutrients between *P. halepensis* and *R. lyciodies*, as *R. lycioides* shrubs growing in the afforested plantation showed higher growth ring $\delta^{13}C$ values and lower radial growth than those in open woodlands, which indicates lower stomatal conductance and impaired water relations in the former. *R. lycioides* shrubs growing near *P. halepensis* trees in the afforested stands were forced to rely on more ephemeral and superficial soil water, which reduced their stomatal conductance (higher leaf $\delta^{18}O$ values) and interfered with nutrient uptake (lower foliar N and P concentrations and more negative leaf $\delta^{15}N$). However, the intense competition for soil resources between *P. halepensis* and *R. lycioides* was more evident in a wet spring (May 2007) than in a dry spring when all the shrubs were severely water stressed irrespective of their distance from the nearest tree (May 2008). Similarly, radial growth differences between *R. lycioides* shrubs growing in afforested vs. open woodland stand types were smaller during very dry years.

In conclusion, contrasting water use patterns among co-occurring plant species in this semiarid environment may allow species coexistence through temporal and spatial partitioning of strongly limiting water resources, which may foster total resource consumption and ecosystem productivity. However, strong intra- and inter-specific competition for water does occur in dense *P. halepensis* afforested plantations, and may be responsible for poor tree growth and slow colonization by native species. Silvicultural practices like thinning can help to reduce intra- and inter-specific competition for water, and may facilitate colonization of afforested stands by late-successional plant species. Stable isotope methodologies are very useful in ecophysiological studies investigating water dynamics in semiarid plant communities, and can help managers to select and adopt appropriate silvicultural practices.

CAPÍTULO 1

INTRODUCCIÓN GENERAL

INTRODUCCIÓN

Antecedentes

Las zonas áridas (o "drylands") incluyen zonas con clima árido, semiárido y seco-subhúmedo y se extienden por un amplio territorio llegando a ocupar alrededor del 41% de la superficie terrestre del planeta (Millennium-Ecosystem-Assessment 2005). Son zonas secas, en las que la evapotranspiración potencial media supera a la precipitación media anual, y por tanto son muy vulnerables a una ulterior intensificación de la sequía (Millennium-Ecosystem-Assessment 2005). Un incremento de la aridez o una mala gestión por parte del hombre podría tener consecuencias irreversibles en estos ecosistemas, tales como la pérdida de la biodiversidad y la desertificación. Se ha visto que la conservación de la riqueza de especies es crucial para preservar las funciones asociadas a los ciclos del C y del N de estos ecosistemas y el mantenimiento de la biodiversidad puede ayudar a amortiguar los efectos previsiblemente negativos del cambio climático y de la desertificación en zonas áridas (Maestre *et al.* 2012).

El SE ibérico es la zona más árida de Europa, con clima mediterráneo semiárido, una precipitación media anual de menos de 350 mm en todo el territorio y una temperatura media anual de entre 16 y 18 ºC en la costa y de entre 12 y 14 ºC en zonas de montaña (Machado *et al.* 2011). La precipitación tiene un fuerte componente aleatorio y es muy irregular. Los modelos de cambio climático para esta zona predicen un descenso de la precipitación y una tendencia hacia periodos secos más largos con un aumento de la variabilidad inter-anual de la cantidad de lluvia y un desplazamiento de la máxima de lluvias de otoño a invierno (Machado *et al.* 2011). Todo esto, sumado al aumento de las temperaturas, podría producir una intensificación de la aridez que puede afectar de manera distinta a las diferentes especies vegetales.

A escala global también se predice un probable aumento de las áreas que se verán afectadas por la sequía (IPCC 2007). Por este motivo, entender los procesos subyacentes que permiten la supervivencia de las especies y el mantenimiento de las comunidades vegetales en ecosistemas áridos y semiáridos es fundamental para la conservación de éstas áreas y para la prevención de la desertificación, pero también es importante para entender el modo en el que las comunidades vegetales se pueden

organizar para adaptarse a las nuevas condiciones de aridez, así como para ayudar en la toma de decisiones de manejo forestal.

Las repoblaciones de *Pinus halepensis* Mill. pueden ser especialmente vulnerables al cambio climático y a la intensificación de la aridez. Estas repoblaciones ocupan vastas áreas del SE ibérico semiárido. Se implantaron con el objetivo inicial de prevenir la erosión y la desertificación a corto plazo y pensando que posteriormente serían colonizadas por otras especies nativas dando así lugar a procesos de sucesión hacia comunidades más naturalizadas (Cortina *et al.* 2011). Sin embargo, esto no ha sido así en muchos casos, y como resultado de esa política encontramos en la actualidad masas forestales monoespecíficas con una alta densidad de pinos que muestran escaso crecimiento y que son muy vulnerables a las plagas y a los incendios (Maestre & Cortina 2004).

Diferenciación ecológica e interacciones planta-planta en ecosistemas semiáridos

En ambientes semiáridos Mediterráneos el agua es el principal factor limitante para la vegetación, y la disponibilidad de los recursos del suelo (agua y nutrientes) muestra una gran variabilidad temporal y espacial. Las plantas perennes dominantes pueden crear cambios estructurales, de tipo micro-climático y de fertilidad del suelo, de modo que pueden forman las llamadas "islas de fertilidad" entorno a las cuales se genera una intensa actividad biológica (Pugnaire, Armas & Maestre 2011). De este modo las plantas se organizan y coexisten en manchas de vegetación. La gran variabilidad espacial y temporal en la disponibilidad de los recursos en estos ecosistemas limitados por el agua proporciona una oportunidad para la diferenciación ecológica (Chesson *et al.* 2004). Las especies vegetales que coexisten en estas comunidades pueden adoptar estrategias complementarias de utilización de los recursos (Arndt 2006, Peñuelas, Terradas & Lloret 2011), llegando de este modo a optimizar la captura total de los recursos y la productividad primaria de estos ecosistemas (Hooper 1998, Hooper *et al.* 2005, O'Connor, Haines & Snyman 2001). Por este motivo no es de extrañar que las zonas áridas den cobijo a numerosos endemismos y comprendan el 20% de los centros de diversidad vegetal del planeta (Maestre *et al.* 2012). Estos centros de diversidad vegetal han sido identificados a escala global por la Unión Internacional para la Conservación de la Naturaleza (UICN) y por la Asociación de la Defensa de la

Naturaleza (WWF – US) debido a su especial relevancia ecológica, puesto que comprenden una gran diversidad de especies vegetales con más de un 10% de endemismos y dan cobijo a más de 1000 especies de plantas vasculares. El SE ibérico también es una región con alta biodiversidad vegetal y más de la mitad de las especies de plantas vasculares que se encuentran en toda la Península Ibérica se concentran en esta zona (Carrión *et al.* 2010).

El principal reto para las plantas en ecosistemas áridos y semi-áridos es la captación y aprovechamiento de los recursos del suelo, que son limitados y se presentan frecuentemente en forma de pulsos debido a las fuertes variaciones intra- e inter-anuales en la cantidad de lluvia (Noy-Meir 1973). Las especies vegetales que coexisten en estos ecosistemas pueden desarrollar distintos mecanismos y estrategias de utilización del agua lo que permite su segregación en nichos ecohidrológicos (Silvertown *et al.* 1999). Las especies vegetales coexistentes pueden diferir considerablemente en su capacidad para aprovechar los pulsos de agua y para soportar periodos secos (Goldberg & Novoplansky 1997) debido por ejemplo a diferencias en sus patrones de enraizamiento (Ehleringer *et al.* 1991, Schwinning & Ehleringer 2001). Las distintas especies coexistentes también pueden diferir en cuanto a su estrategia para afrontar el estrés hídrico (Valladares *et al.* 2004): pueden tolerar el estrés hídrico y la deshidratación (estrategia anisohídrica), pueden evitar el estrés mediante una estrategia isohídrica o pueden escapar al estrés completando su ciclo vital antes de la llegada del estrés hídrico o entrando en dormancia vegetativa. Varios estudios han encontrado múltiples estrategias de utilización del agua por parte de especies coexistentes que son igualmente exitosas en ecosistemas semiáridos (e.g. Ackerly 2004, Jacobsen *et al.* 2008, Peñuelas *et al.* 2011, West *et al.* 2012). Estas estrategias se reflejan en una serie de rasgos fisiológicos de las plantas (e.g. patrón de enraizamiento, fenología de la hoja, potencial hídrico de los tallos, resistencia a la cavitación, tasa fotosintética, concentración de nutrientes en hoja…) que pueden medirse para poder identificar dichas estrategias y ayudar a entender la diversidad funcional de las comunidades vegetales. La identificación de grupos o tipos funcionales discretos es válido como esfuerzo de síntesis para ayudar a entender cómo se organizan las comunidades vegetales, pero la realidad es que suele haber muy poca discontinuidad entre estos grupos (Ackerly 2004). Algunos estudios evidencian que las estrategias de las plantas se distribuyen de manera

continua a lo largo de unos ejes que están determinados por los rasgos fisiológicos de las plantas (Angert *et al.* 2009, West *et al.* 2012, Wright *et al.* 2004). Entender la ecología funcional de las distintas especies vegetales coexistentes e identificar sus estrategias de utilización de los recursos es fundamental para predecir la respuesta de las plantas ante cambios en los factores externos que puedan alterar el equilibrio actual de las comunidades vegetales (Arndt 2006).

Junto a una diferenciación en las estrategias de utilización de los recursos por parte de las plantas, en las manchas de vegetación de los ecosistemas áridos y semi-áridos las plantas interaccionan entre sí pudiendo dar lugar a relaciones de competencia y/o facilitación. Las interacciones planta-planta son fundamentales a la hora de estructurar las comunidades vegetales en ecosistemas áridos y semiáridos (Fowler 1986). Las relaciones de competencia por los recursos o de facilitación son muy comunes y pueden ocurrir simultáneamente (Callaway & Walker 1997). El resultado neto de estas interacciones es complejo y va a depender de varios factores, entre ellos variaciones temporales en la disponibilidad de los recursos (Tielbörger & Kadmon 2000) y cambios en la edad y etapa vital de las plantas (Miriti 2006, Schiffers & Tielbörger 2006). En ecosistemas semiáridos la competencia por los recursos del suelo (agua y nutrientes) es muy importante y su intensidad va a depender de la identidad de las especies vegetales que interaccionan entre sí (Armas & Pugnaire 2011). Sin embargo, también se pueden dar relaciones de facilitación que pueden llegar a prevalecer a nivel de comunidad (Pugnaire, Armas & Valladares 2004). Entre los procesos de facilitación que se pueden dar entre plantas encontramos el sombreo y la mejora de las condiciones micro-climáticas (Gómez-Aparicio *et al.* 2004, Maestre *et al.* 2001), la mejora de la disponibilidad de agua mediante redistribución hidráulica por las raíces de algunas especies (Dawson 1993, Egerton-Warburton, Querejeta & Allen 2007, Filella & Peñuelas 2003a, Prieto, Armas & Pugnaire 2012) y el incremento del contenido de nutrientes del suelo en la rizosfera de las especies facilitadoras (Armas *et al.* 2012, Pugnaire *et al.* 2004).

Dentro de la teoría ecológica de las interacciones entre plantas, se ha formulado la hipótesis del gradiente del estrés (Bertness & Callaway 1994) que propone que la frecuencia de las interacciones positivas entre plantas, de tipo facilitativo, aumenta en ecosistemas con un alto grado de estrés ambiental, como ocurre en las zonas áridas. Sin

embargo, pese a que muchos estudios parecen apoyarla, los resultados de otros trabajos contradicen esta hipótesis que sigue siendo objeto de debate (ver revisiones de Brooker *et al.* 2008 y Maestre *et al.* 2009). Puesto que las relaciones de competencia y facilitación entre plantas ocurren simultáneamente, es necesario tener en cuenta la importancia relativa de estos procesos, y no sólo su intensidad, para poder determinar el balance neto de estas interacciones (Brooker & Kikividze 2008).

Es fundamental conocer los procesos ecofisiológicos específicos que resultan afectados por las interacciones entre plantas (a nivel intra- o inter-específico) para poder entender la importancia relativa de las relaciones de competencia o facilitación y cómo éstas afectan a la organización de las comunidades vegetales. Además, esto ayudaría a predecir cómo estas interacciones pueden verse afectadas por cambios ambientales y climáticos o cómo pueden variar con la edad de las plantas, y aportaría luz en el actual debate sobre el balance neto de las interacciones entre plantas. Por todo ello, es oportuno el empleo de técnicas que permitan estudiar en detalle los procesos ecofisiológicos que se ven afectados por las interacciones entre plantas. Las técnicas basadas en isótopos estables pueden resultar muy útiles, pues pueden proporcionar una información integrada en el tiempo de los procesos de intercambio de gases en hoja que ocurren en la planta durante su crecimiento.

Dendroecología

La dendroecología es la ciencia que pretende extraer de las secuencias de anillos de crecimiento de los árboles algún tipo de información ambiental (Schweingruber 1996). En conjunción con las medidas de la composición isotópica de los anillos de crecimiento pueden proporcionar registros retrospectivos de largo alcance sobre la actividad fisiológica de las plantas en respuesta a su medio cambiante. Las curvas de crecimiento de los árboles también pueden reflejar relaciones de competencia y/o facilitación entre plantas (Schweingruber 1996).

En las coníferas, los anillos de crecimiento están básicamente formados por traqueidas, que cambian de tamaño a lo largo de la estación de crecimiento: las formadas en primavera son más anchas y con pared más fina y las formadas al final de la estación de crecimiento son más pequeñas, con forma aplanada y pared más gruesa. Esto hace que los anillos se muestren como una sucesión de bandas claras y oscuras que

se corresponden respectivamente con la madera temprana (formada al inicio de la estación de crecimiento) y tardía (formada al final de la estación de crecimiento) de cada año. En angiospermas, los anillos están formados por diferentes tipos de células y su proporción y aspecto van cambiando a lo largo de la estación de crecimiento por lo que se hace posible la identificación de los anillos de crecimiento. El aspecto externo de los anillos va a depender del tipo de madera que presente cada especie (de anillo poroso, difuso o semiporoso).

A pesar de su potencial para predecir los impactos del cambio climático, los estudios dendroecológicos son escasos en la región mediterránea (Cherubini *et al.* 2003). Esto se debe a que la identificación y datación de los anillos de crecimiento de las especies de plantas que crecen en esta región no es sencilla. En el área mediterránea existe una elevada variabilidad climática estacional y el crecimiento de las plantas está limitado tanto por las bajas temperaturas de invierno como por la sequía de verano (Cherubini *et al.* 2003). Este "doble estrés" hace que en algunos años puedan aparecer dobles anillos o anillos falsos (causados por la interrupción del crecimiento debido a factores como la sequía y su posterior reanudación dentro de la misma estación de crecimiento, de Luis *et al.* 2011), o incluso puede hacer que no llegue a formarse ningún anillo. Sin embargo, la datación de los anillos de crecimiento en la región mediterránea es posible, aunque difícil (Cherubini *et al.* 2003).

Las técnicas de isótopos estables

La aplicación de las técnicas de isótopos estables en estudios de ecología vegetal ha tenido un crecimiento exponencial en los últimos años (Resco *et al.* 2011). El análisis de la composición isotópica en oxígeno e hidrógeno ($\delta^{18}O$ y δD, respectivamente) del agua extraída de tejidos leñosos permite estudiar las fuentes de agua utilizadas por las plantas (Ehleringer & Dawson 1992). Por otra parte, las composición isotópica en oxígeno y carbono ($\delta^{18}O$ y $\delta^{13}C$, respectivamente) de la materia orgánica de la planta proporcionan una medida integrada en el tiempo de los procesos de intercambio de gases que ocurren a nivel foliar durante el periodo de crecimiento del tejido vegetal estudiado (Barbour 2007, Dawson *et al.* 2002, Farquhar, Ehleringer & Hubick 1989). Un enfoque basado en métodos isotópicos es de tipo no destructivo y supone una inversión en tiempo y esfuerzo considerablemente menor que

el que requeriría un enfoque basado exclusivamente en métodos ecofisiológicos convencionales, lo cual permite aumentar considerablemente el número de plantas muestreadas e incluir un mayor número de especies y localidades de estudio.

Sin embargo, las técnicas de isótopos estables deben usarse con cautela y para su correcta interpretación deben respetarse los supuestos de los modelos que los describen. Además es conveniente acompañar las medidas isotópicas de otras medidas ecofisiológicas (e.g. crecimiento, intercambio de gases en hoja, potencial hídrico). Estudios recientes han alertado sobre la falta de respuesta de la composición isotópica foliar en algunas situaciones (Roden & Farquhar 2012) y sobre la necesidad de profundizar en el estudio de otros factores que hasta ahora no habían sido considerados explícitamente pero que en algunos casos pueden afectar significativamente a la composición isotópica de la planta, como por ejemplo la conductancia del mesófilo (Ferrio *et al.* 2012, Warren & Adams 2006) o el fraccionamiento asociado a la carga y transporte de compuesto orgánicos en el floema (Offermann *et al.* 2011).

Los isótopos estables son átomos de un mismo elemento que contienen distinto número de neutrones y que no decaen con el tiempo (escala de tiempo geológica). Al tener una masa atómica distinta, se ven afectados por los procesos dependientes de masa atómica que producen un fraccionamiento o sesgo en la composición isotópica de un elemento. Ocurren dos principales tipos de fraccionamiento (Dawson *et al.* 2002):

- Fraccionamiento de equilibrio: en reacciones que conllevan cambio de fase (como de líquido a vapor). Normalmente estas reacciones no se completan u ocurren en sistemas abiertos, dando lugar a un reparto desigual de los distintos isótopos entre fases por lo que estos procesos son en realidad de no-equilibrio. Son reacciones dependientes de la temperatura.

- Fraccionamiento cinético: reacciones unidireccionales cuyas tasas de reacción dependen de la masa atómica. En los sistemas biológicos suelen ser reacciones catalizadas por algún enzima que hace discriminación entre isótopos.

De este modo, la composición isotópica de un elemento puede funcionar como indicador de estos procesos. En estudios de ecología vegetal se miden principalmente $\delta^{18}O$ y $\delta^{13}C$, especialmente en ecosistemas áridos y semi-áridos, por la información que proporcionan sobre las relaciones carbono-agua en la planta.

La composición isotópica de un elemento se mide por espectrometría de masas y se expresa en notación δ (en partes por mil, ‰) como el ratio isotópico de la muestra respecto al ratio isotópico de un estándar internacionalmente reconocido:

$$\delta^{XX}\mathrm{E} = 1000 \cdot \left(\frac{\mathrm{R}_{\mathrm{muestra}}}{\mathrm{R}_{\mathrm{estándar}}} - 1 \right)$$

Donde E es el elemento de interés y R es el ratio de la abundancia del isótopo más pesado respecto al más ligero.

La composición isotópica del agua de los tallos, del suelo y de la lluvia

El análisis de la abundancia natural de los isótopos estables de hidrógeno (δD) y oxígeno ($\delta^{18}O$) en agua permite distinguir qué fuentes de humedad utilizan las plantas (Casper, Schenk & Jackson 2003, Dawson *et al.* 2002, Ehleringer & Dawson 1992, Williams & Ehleringer 2000). Salvo algunas excepciones (Ellsworth & Williams 2007), durante la asimilación del agua del suelo por parte de las raíces no existe fraccionamiento y por tanto la composición isotópica en deuterio y oxígeno del agua del xilema refleja la composición isotópica de la fuente de agua utilizada por la planta (Dawson *et al.* 2002).

En zonas con suelos bien drenados y en ausencia de agua subterránea, el agua de lluvia es la principal fuente de humedad para las plantas. La composición isotópica de la precipitación depende fuertemente de la temperatura de condensación de las gotas de lluvia (Dansgaard 1964), lo que hace que la composición isotópica de la precipitación pueda variar mucho de una estación a otra y también entre años. En el SE ibérico, la variabilidad estacional en los valores de δD y $\delta^{18}O$ del agua de lluvia se debe también al distinto origen de los frentes de lluvia, que pueden proceder del Atlántico o del Mediterráneo y que presentan diferente composición isotópica (Vandenschrick *et al.* 2002). Sin embargo, el ratio entre los valores de δD y $\delta^{18}O$ del agua de lluvia se mantiene relativamente constante si la precipitación no se somete a ulteriores procesos de evaporación (Dansgaard 1964). La relación constante entre el δD y el $\delta^{18}O$ del agua de lluvia a escala global fue descrita por primera vez por (Craig 1961) y se representa gráficamente como la Línea Meteórica Globlal ("Global Meteoric Water Line", GMWL) según la ecuación:

$$\delta D = 8 \cdot \delta^{18}O + 10$$

La existencia de Líneas Meteóricas Locales con pendientes más bajas indican ulteriores procesos de fraccionamiento, como por ejemplo la evaporación que pueden sufrir las gotas de lluvia durante su caída en ambientes muy secos (Dansgaard 1964). Puesto que la relación entre del agua de lluvia puede variar localmente, es importante construir la Línea Meteórica Local de cada zona de estudio.

Ya en el suelo, el agua remanente se enriquece en los isótopos más pesados (D y ^{18}O) por un efecto de fraccionamiento isotópico evaporativo. Este enriquecimiento hace que la composición isotópica en deuterio y oxígeno del agua del suelo se desvíe respecto a la Línea Meteórica Local. El grado de evaporación que sufre una masa de agua puede estimarse calculando su *d*-excess (Dawson & Simonin 2011):

$$d\text{-excess} = \delta D - 8 \cdot \delta^{18}O$$

Con la evaporación, el agua remanente disminuye su valor de *d*-excess.

En sistemas áridos, se pueden desarrollar marcados gradientes verticales en la composición isotópica en hidrógeno y oxígeno del agua del suelo durante periodos prolongados sin lluvia (Barnes & Allison 1983). Este gradiente vertical se distribuye a lo largo del perfil del suelo puesto que los procesos de fraccionamiento isotópico evaporativo son más intensos en superficie y van disminuyendo con la profundidad (Barnes & Turner 1998). De este modo, el agua de los horizontes más superficiales está más evaporada y enriquecida en los isótopos más pesados de hidrógeno y oxígeno que el agua de los horizontes más profundos, donde el efecto de la evaporación es menor.

Puesto que en ecosistemas semiáridos puede formarse este marcado gradiente vertical en la composición isotópica del agua del suelo, esto nos permite discernir entre especies de enraizamiento profundo y superficial (Filella & Peñuelas 2003b, Stratton, Goldstein & Meinzer 2000, Williams & Ehleringer 2000). En este sentido puede ser útil el cálculo de la diferencia entre el valor de *d*-excess de la precipitación local y el *d*-excess del agua del xilema ($d\text{-excess}_{lluvia} - d\text{-excess}_{xilema}$). Esta diferencia será mayor cuanto mayor sea la proporción de agua superficial y evaporada que usen las plantas, y por tanto puede considerarse como un indicador de la profundidad a la que las plantas extraen el agua (Dawson & Simonin 2011). Sin embargo, se ha visto recientemente que algunas plantas xerofíticas y halofíticas pueden discriminar frente al deuterio durante la

absorción del agua (Ellsworth & Williams 2007). Por este motivo, en ecosistemas salinos o áridos el valor de $\delta^{18}O$ del agua del xilema puede constituir un mejor indicador de la profundidad de extracción del agua por las plantas que el valor de *d*-excess.

Cuando las plantas extraen agua de varios horizontes a la vez, la interpretación de los datos isotópicos resulta más complicada. Sin embargo sigue siendo posible calcular la proporción de agua absorbida por la planta en diferentes capas del suelo y subsuelo mediante la utilización de modelos de mezcla isotópica (Phillips & Gregg 2003).

El análisis de δD y $\delta^{18}O$ del agua del xilema no sólo ayuda a diferenciar entre las fuentes de agua que utilizan las distintas especies vegetales, sino que también pueden usarse para examinar variaciones estacionales en la profundidad de absorción de agua, diferencias entre individuos jóvenes y adultos, fenómenos de competencia y de facilitación específicamente relacionados con el agua (por ejemplo, redistribución hidráulica por las raíces), etc. En este caso, la novedad de las técnicas de isótopos estables reside en que nos proporcionan información sobre la actividad y funcionalidad de las raíces, cosa que no siempre es posible evaluar con el mero análisis visual de sus patrones de enraizamiento.

La composición isotópica en carbono de la materia orgánica de la planta

Durante la asimilación del CO_2 atmosférico la planta discrimina frente al isótopo más pesado del carbono de tal manera que la composición isotópica de carbono de la planta es muy distinta a la del CO_2 atmosférico (la mayoría de las plantas C3 presentan valores entre -30‰ y -22‰ mientras que el valor del CO_2 atmosférico en la actualidad es aproximadamente -8‰). Esta discriminación puede calcularse según la siguiente ecuación (Seibt *et al.* 2008):

$$\Delta^{13}C = \frac{\delta^{13}C_{atm} - \delta^{13}C_p}{1 + \delta^{13}C_p}$$

donde $\delta^{13}C_{atm}$ y $\delta^{13}C_p$ equivalen respectivamente a la composición isotópica de carbono del CO_2 atmosférico y de la materia orgánica de la planta. Hay que tener en cuenta que los valores que se dan en unidades de "por mil" deben ser divididos por 1000 cuando aparecen en términos tales como $(1 + \delta^{13}C_p)$.

La discriminación frente al ^{13}C que ocurre en la planta se debe a la diferente afinidad de los isótopos del carbono por las enzimas que catalizan el proceso de carboxilación y a la distinta difusividad que tiene el $^{13}CO_2$ frente al $^{12}CO_2$. De este modo, los valores de $\delta^{13}C_p$ responden a cambios en la actividad fotosintética de la planta y a variaciones en la conductancia estomática de la hoja. En el material vegetal de las plantas C3, los valores de $\Delta^{13}C$ se correlacionan positivamente con el ratio entre la presión parcial de CO_2 en los espacios intercelulares de la hoja y en el aire (c_i/c_a) durante la fotosíntesis (Farquhar *et al.* 1989). Por el contrario, la composición isotópica del carbono ($\delta^{13}C$) de la planta está lineal e inversamente correlacionada con c_i/c_a. Esta relación se describe en el modelo propuesto por (Farquhar *et al.* 1989):

$$\Delta^{13}C = a + (b - a) \cdot \frac{c_i}{c_a}$$

donde "a" equivale al fraccionamiento asociado a la difusión del CO_2 a través de los estomas ($^{13}CO_2$ tiene una menor difusividad que $^{12}CO_2$; este factor equivale a 4.4‰, (O'Leary 1981) y "b" al fraccionamiento asociado a la diferente capacidad de reacción del $^{13}CO_2$ y del $^{12}CO_2$ con la Rubisco y a la transferencia interna del CO_2 (este factor es aproximadamente 27‰; Farquhar & Richards 1984).

Puesto que c_i depende de la conductancia estomática y de la demanda de CO_2 en los sitios de carboxilación (y por tanto de la actividad fotosintética), los cambios en los valores de $\delta^{13}C$ de los tejidos vegetales también son proporcionales a los cambios en los valores de eficiencia intrínseca en el uso del agua de la planta ("intrinsic water use efficiency", WUE_i), que se calcula como el ratio entre la tasa fotosintética neta (A) y la conductancia estomática al vapor de agua (g_s; Ehleringer, Hall & Farquhar 1993):

$$WUE_i = \frac{A}{g_s} = \frac{(c_a - c_i)}{1.6}$$

Usando las dos ecuaciones anteriores se puede obtener una medida integrada en el tiempo de los valores de eficiencia intrínseca en el uso del agua de la planta a partir de los valores de $\Delta^{13}C$ del material vegetal (Seibt *et al.* 2008):

$$WUE_i = \frac{A}{g_s} = \frac{c_a}{1.6}\left(\frac{b - \Delta^{13}C}{b - a}\right)$$

Los valores de $\delta^{13}C_p$ están positivamente correlacionados con los valores de WUE_i y reflejan por tanto la interacción entre las relaciones hídricas y el balance de carbono de la planta, resultando así muy útiles como integradores del funcionamiento de la planta (Klein *et al.* 2005, Warren, McGrath & Adams 2001). Numerosos estudios han encontrado que los valores de $\delta^{13}C$ de la planta se relacionan con factores ambientales que modifican directa o indirectamente la tasa fotosintética y/o la conductancia estomática. Por tanto, las variables ambientales relacionadas con la disponibilidad de agua o el déficit de presión de vapor entre la hoja y el aire, también pueden afectar al $\delta^{13}C$. Cambios en la composición isotópica de carbono de las plantas se han relacionado con variaciones en la disponibilidad de agua del suelo, en la cantidad de lluvia, en la temperatura, en el déficit de presión de vapor de agua, en la humedad relativa, en la disponibilidad de nutrientes y en la irradiancia (Ferrio *et al.* 2003, Keitel *et al.* 2006, Klein *et al.* 2005, Saurer *et al.* 1997c, Saurer, Siegenthaler & Schweingruber 1995, Stewart *et al.* 1995, Warren *et al.* 2001).

Recientemente se ha puesto en evidencia la complejidad de la interpretación de los valores de $\delta^{13}C$ de la planta y se recomienda acompañarlos de otras medidas fisiológicas. El modelo de Farquhar *et al.* (1989) es una simplificación y no considera explícitamente los fraccionamientos asociados a la conductancia del mesófilo y a la fotorespiración (para una explicación más detallada ver Seibt *et al.* 2008) cuyo efecto puede llegar a ser importante en algunos casos (Warren & Adams 2006). Otros procesos de fraccionamiento ocurren después de la formación de los primeros productos de la fotosíntesis y pueden llegar a ser importantes cuando los valores de $\delta^{13}C$ no se miden en hoja sino en otros tejidos como raíces o anillos de crecimiento. Entre los procesos en los que puede haber una discriminación del ^{13}C están la carga y transporte de compuestos orgánicos en el floema, la respiración y la fotorespiración (Badeck *et al.* 2005, Ghashghaie *et al.* 2003, Hobbie & Werner 2004, Offermann *et al.* 2011). Además estos procesos podrían variar con la especie considerada y con las condiciones ambientales.

La complejidad de la interpretación de los valores de $\delta^{13}C$ de la planta reside también en el hecho de que estos valores se ven influidos indistintamente por cambios en la tasa fotosintética y/o en la conductancia estomática. En este sentido, el análisis conjunto de los valores de $\delta^{18}O$ de la materia orgánica de la planta puede ayudar a discernir entre los efectos independientes de A y g_s sobre el $\delta^{13}C$ de la planta, puesto

que los valores de $\delta^{18}O$ se ven influidos por g_s pero no responden a cambios en A (Grams *et al.* 2007, Roden & Farquhar 2012, Scheidegger *et al.* 2000).

La composición isotópica en oxígeno de la materia orgánica de la planta

La composición isotópica en oxígeno de los tejidos vegetales ($\delta^{18}O_p$) se ve influida por la composición isotópica de la fuente de agua utilizada por la planta ($\delta^{18}O_s$) pero también por los procesos evaporativos que enriquecen isotópicamente el agua de la hoja (Barbour 2007). Los átomos de oxígeno que se incorporan a las moléculas orgánicas durante la fotosíntesis reflejan la composición isotópica del agua de la hoja (Roden, Lin & Ehleringer 2000). Aunque el CO_2 también proporciona átomos de oxígeno al metabolismo de los carbohidratos éste no influye en la composición isotópica en oxígeno de la materia orgánica puesto que entra en equilibrio con el agua de la hoja y todos los átomos de oxígeno del CO_2 se intercambian con los del agua antes de la síntesis de carbohidratos (DeNiro & Epstein 1979).

El agua que absorben las raíces de la planta llega por el xilema a la hoja y allí sufre un enriquecimiento isotópico debido a un fraccionamiento de tipo evaporativo. El enriquecimiento isotópico en oxígeno que sufre el agua de la hoja en los lugares de evaporación ($\Delta^{18}O_e = \delta^{18}O_e - \delta^{18}O_s$) se describe en el modelo propuesto por Craig & Gordon (1965) y adaptado por Dongmann *et al.* (1974):

$$\Delta^{18}O_e = \varepsilon_k + \varepsilon_e + (\Delta^{18}O_v - \varepsilon_k)\frac{e_a}{e_i}$$

donde ε_e es el factor de fraccionamiento (de equilibrio) debido al cambio de fase de líquido a vapor y depende de la temperatura (9,5‰ a 20ºC; Barbour 2007, Barbour, Walcroft & Farquhar 2002); ε_k es el factor de fraccionamiento (cinético) debido a la difusión del vapor de agua desde la hoja a la atmósfera a través de los estomas y de la capa límite (se puede calcular a partir de las conductancias estomática y de la capa límite, según propone Farquhar, Barbour & Henry 1998, ver también Barbour 2007); $\Delta^{18}O_v$ es el enriquecimiento isotópico del vapor de agua comparado con la fuente de agua y e_a y e_i son respectivamente las presiones de vapor en el aire y en los espacios intercelulares de la hoja. Normalmente, cuando las condiciones de mezcla de aire son buenas, $\Delta^{18}O_v$ se aproxima a "- ε_e" y entonces $\Delta^{18}O_e$ es proporcional a $1-e_a/e_i$. Puesto

que un incremento de la transpiración produce un enfriamiento de la hoja, esto disminuye la presión de vapor en los espacios intercelulares de la hoja (e_i) y hace aumentar el ratio e_a/e_i, que está lineal e inversamente relacionado con $\Delta^{18}O_e$ y $\delta^{18}O_e$. Por lo tanto, cuando aumentan la conductancia estomática y la transpiración de la hoja, los valores de $\Delta^{18}O_e$ y $\delta^{18}O_e$ tienden a disminuir.

El modelo de Dongmann *et al.* (1974) describe bien la tendencia de la composición isotópica del agua de la hoja en los lugares de evaporación, pero cuando se considera todo el agua de la hoja, a este modelo se le deben sumar las desviaciones producidas por el efecto Péclet (Barbour 2007). El efecto Péclet ($\wp$) consiste en una atenuación del enriquecimiento isotópico del agua de la hoja debido a la oposición entre el movimiento convectivo del agua menos enriquecida del xilema frente a la difusión en sentido opuesto del agua isotópicamente enriquecida proveniente de los lugares de evaporación. De este modo, el enriquecimiento isotópico promedio de la lámina de agua de la hoja ($\Delta^{18}O_{lw}$) se describe según el siguiente modelo (Farquhar & Lloyd 1993):

$$\Delta^{18}O_{lw} = \frac{\Delta^{18}O_e(1-e^{-\wp})}{\wp}$$

El efecto Péclet aumenta con la longitud efectiva del recorrido que debe realizar el agua a través del mesófilo de la hoja (L) y con la tasa de transpiración (Barbour 2007). Ferrio *et al.* (2009) encontraron que este efecto puede depender también del estado hídrico de la planta, pues vieron que L aumenta en plantas con un mayor estrés hídrico.

El enriquecimiento evaporativo que ocurre en el agua de la hoja se traslada a la materia orgánica debido al intercambio de los átomos de oxígeno de los grupos carbonilo con el agua. Durante este proceso ocurre un fraccionamiento de tipo bioquímico que hace que los valores de $\delta^{18}O$ de la celulosa que se forma en los tejidos fotosintéticos de la planta sean un 27‰ mayores que los del agua de la hoja (Sternberg, Anderson & Morrison 2003). Del mismo modo, la sacarosa que es transportada en el floema refleja los valores de $\Delta^{18}O_{lw}$ aunque enriquecidos en un 27 ‰ (Barbour *et al.* 2000b).

Por todo esto, los valores de $\Delta^{18}O$ del material foliar ($\Delta^{18}O_p = \delta^{18}O_p - \delta^{18}O_s$) dependen también del gradiente de presión parcial de vapor de agua que existe entre el

aire y los espacios intercelulares de la hoja (e_a/e_i; Barbour 2007, Farquhar *et al.* 1998) y por tanto, van a depender del déficit de presión de vapor de agua del aire, de la humedad relativa, de la humedad del suelo y de la transpiración de la planta. Diferentes estudios han encontrado que los valores de $\delta^{18}O$ o $\Delta^{18}O$ foliar también disminuyen en respuesta a un incremento de la conductancia estomática (Barbour *et al.* 2000a, Farquhar, Cernusak & Barnes 2007, Grams *et al.* 2007). Sin embargo, el salto de la transpiración a la conductancia estomática no es inmediato y a la hora de interpretar los valores de $\Delta^{18}O_p$ debe tenerse en cuenta que puede haber variaciones en la transpiración de la hoja debidas a diferencias en el déficit de presión de vapor de agua del aire o en la humedad relativa que no se deban a cambios en la conductancia estomática de la planta (Roden & Farquhar 2012).

Durante la formación de celulosa en los tejidos no-fotosintéticos de la planta se producen ulteriores procesos de fraccionamiento. Esto es especialmente importante cuando se mide la composición isotópica en oxígeno de los anillos de crecimiento de las plantas ($\delta^{18}O_R$). Para poder formar celulosa, la sacarosa transportada por el floema desde las hojas hasta el tejido de destino se rompe dando lugar a hexosas fosfato. Durante este proceso un 20% de los átomos de oxígeno se intercambian con las moléculas de agua del medio (Farquhar *et al.* 1998). Pero además, una proporción variable de estas hexosas fosfato pasa por el ciclo "fútil" de las triosas fosfato en donde un 60% de los átomos de oxígeno es intercambiado (Hill *et al.* 1995). Como resultado final de ambos procesos, se estima que cerca del 40% de los átomos de oxígeno que formarán la celulosa se intercambia con el agua en los tejidos no-fotosintéticos de la planta (Roden *et al.* 2000).

Puesto que es elevada la proporción de átomos de oxígeno que se intercambian con el agua del xilema durante la formación de la celulosa en los anillos de crecimiento, los valores de $\delta^{18}O_R$ reflejan tanto a composición isotópica del agua de lluvia (cuando ésta es la principal fuente de agua) como los procesos evaporativos que se dan en la hoja. La importancia relativa de cada uno de estos factores en $\delta^{18}O_R$ podrá variar en cada caso.

En algunos casos, el efecto de enriquecimiento isotópico de tipo evaporativo que se da en la hoja puede prevalecer sobre la influencia de la composición isotópica de la fuente de agua en $\delta^{18}O_R$ (Ferrio & Voltas 2005) y los valores de $\delta^{18}O_R$ pueden reflejar

los valores de $\delta^{18}O$ de los primeros productos de la fotosíntesis. Jäggi *et al.* (2003) encontraron una correlación muy significativa entre los valores de $\delta^{18}O$ de la madera y de las acículas de años correspondientes en *Picea abies* (si bien la señal reflejada en la composición isotópica en oxígeno de las acículas era unas 4 veces más fuerte que la que quedaba reflejada en la madera). Gessler *et al.* (2009) también encontraron una buena correspondencia entre los valores de $\delta^{18}O$ del agua de la hoja, de los azúcares transportados en el floema y de los anillos de crecimiento en *Pinus sylvestris*. Además, varios estudios han encontrado que los valores de $\delta^{18}O$ de los anillos de crecimiento se correlacionan con los factores ambientales responsables de producir cambios en los procesos evaporativos de la hoja, incluyendo entre ellos la disponibilidad de agua para la planta durante la estación de crecimiento. De este modo, los valores de $\delta^{18}O_R$ se han relacionado con la precipitación, la humedad relativa y el déficit de presión de vapor (Battipaglia *et al.* 2007, Ferrio & Voltas 2005, Hilasvuori & Berninger 2010, Keitel *et al.* 2006, McCarroll & Loader 2004, Reynolds-Henne, Saurer & Siegwolf 2009, Saurer, Borella & Leuenberger 1997b).

Algunos autores recomiendan que se lleve a cabo la purificación de la celulosa antes de medir los valores de $\delta^{18}O$ del material vegetal (Grams *et al.* 2007, Powers, Pregitzer & Palik 2008, Roden & Farquhar 2012). Esto se debe a que la materia orgánica de la planta está formada por proporciones variables de compuestos orgánicos (taninos, resinas, ceras, aceites…) que tienen diferente composición isotópica y pueden enmascarar las variaciones en los valores de $\delta^{18}O$ debidas a cambios en los procesos evaporativos de la hoja. Además, en el caso de los anillos de crecimiento, la extracción de la celulosa es importante porque después de su síntesis ésta ya no intercambia sus átomos con el agua del medio (Gaudinski *et al.* 2005) y puede conservar intacta su señal isotópica a lo largo de los años. Por el contrario, se ha visto que los valores de $\delta^{13}C_p$ se correlacionan bastante bien cuando se miden en todo el material vegetal y en la celulosa purificada (Grams *et al.* 2007, Powers *et al.* 2008, Roden & Farquhar 2012).

El estudio combinado de $\delta^{13}C$ y $\delta^{18}O$ en la materia orgánica de la planta

La medida conjunta de $\delta^{13}C$ y $\delta^{18}O$ en el material vegetal permite separar los efectos independientes de la tasa fotosintética y de la conductancia estomática sobre los valores de $\delta^{13}C$. Este hecho se debe a que los valores de $\delta^{18}O$ cambian con la

conductancia estomática pero no con la tasa de fijación de carbono (Grams *et al.* 2007, Scheidegger *et al.* 2000). De este modo, los valores de $\delta^{13}C$ y $\delta^{18}O$ permiten diferenciar si la actividad fotosintética de la planta está limitada principalmente por un factor bioquímico o si en cambio sufre una limitación estomática a la difusión del CO_2 atmosférico hacia el interior de la hoja.

El modelo semicuantitativo de Scheidegger *et al.* (2000), modificado por Grams *et al.* (2007), predice que al disminuir la conductancia estomática aumenta el $\delta^{18}O$ de la planta, mientras que la tendencia en los valores de $\delta^{13}C$ dependerá de la respuesta simultánea de la tasa fotosintética. Por ejemplo, cuando la humedad del suelo disminuye suele producirse simultáneamente la disminución de la tasa fotosintética, de la transpiración y de la conductancia estomática de la planta. Según el modelo, en este caso aumentarían los valores de $\delta^{18}O$ de la planta (puesto que disminuyen su transpiración y la conductancia estomática) mientras que los valores de $\delta^{13}C$ no variarían, puesto que la tasa fotosintética disminuiría en paralelo a la conductancia estomática sin que el ratio entre ambos parámetros se viera afectado significativamente. El modelo permite identificar cambios relativos en la tasa fotosintética y en la conductancia estomática, pero aún se necesita avanzar más en el conocimiento de este modelo para poder utilizarlo de forma cuantitativa.

Este método ya ha sido utilizado con éxito en numerosos estudios ecológicos donde se han evaluado variaciones en la fisiología de las plantas tanto a lo largo de gradientes ambientales (Barbour *et al.* 2002, Keitel *et al.* 2006, Sullivan & Welker 2007) como en respuesta a diferente intensidad de la competencia inter-específica por los recursos (Ramírez, Querejeta & Bellot 2009), así como en respuesta a algún tratamiento (Grams *et al.* 2007, Querejeta *et al.* 2006, Querejeta *et al.* 2008) o para comparar la actividad fisiológica de plántulas con distinto origen geográfico cultivadas en condiciones ambientales homogéneas (Voltas *et al.* 2008) .

La medida conjunta de $\delta^{18}O$ y $\delta^{13}C$ en anillos de crecimiento permite hacer inferencias precisas sobre las condiciones ambientales a las que ha estado sometida la planta en el pasado y su respuesta ecofisiológica a las mismas (fuentes de agua utilizadas, eficiencia en el uso del agua, niveles de estrés hídrico, etc.), por lo que se ha aplicado en algunos estudios que han evaluado en el largo plazo las respuestas

ecofisiológicas de las plantas a cambios ambientales (Battipaglia *et al.* 2009, Hilasvuori & Berninger 2010, Saurer, Aellen & Siegwolf 1997a).

Un estudio reciente de Roden & Farquhar (2012) evidenció la necesidad de tener precaución a la hora de utilizar este método en ciertos casos, especialmente cuando existen diferencias entre tratamientos en el déficit de presión de vapor o en la humedad relativa que pueden alterar la transpiración de la hoja y por tanto su composición isotópica en oxígeno, sin alterar necesariamente la conductancia estomática de la hoja.

La composición isotópica en nitrógeno de la materia orgánica de la planta

La composición isotópica en nitrógeno ($\delta^{15}N$) del material vegetal está relacionada con la disponibilidad de nutrientes y de agua para la planta (Tilman 1988). Su interpretación no es sencilla puesto que responde a una combinación de múltiples procesos cuya importancia relativa puede variar de un caso a otro. Los valores de $\delta^{15}N$ de la planta pueden reflejar la utilización de diferentes fuentes de nitrógeno, las asociaciones micorrízicas y las variaciones espaciales y temporales en la disponibilidad y demanda de nitrógeno por parte de la planta (Bai *et al.* 2009, Dawson *et al.* 2002, Högberg 1997).

Las leguminosas y las plantas actinorrízicas presentan asociaciones con bacterias capaces de fijar el N_2 atmosférico. Esta fuente de nitrógeno para la planta tiene una composición isotópica igual a 0‰ y a menudo puede diferenciarse claramente de otras fuentes de nitrógeno presentes en el suelo. Por este motivo, numerosos trabajos han medido la composición isotópica de nitrógeno foliar para estudiar el uso de nitrógeno fijado simbióticamente en casos en los que intervienen plantas leguminosas o actinorrízicas. Por ejemplo, (Bai *et al.* 2009) relacionaron los valores de $\delta^{15}N$ foliar de dos arbustos perennifolios con un índice de proximidad a un árbol leguminoso (*Prosopis glandulosa*), encontrando valores más próximos a 0‰ en proximidad a este árbol e indicando por tanto la influencia de la leguminosa en la utilización de nitrógeno por parte de plantas no-leguminosas.

Los valores de $\delta^{15}N$ foliar también se han utilizado con éxito para determinar diferencias en las fuentes de nitrógeno utilizadas por las plantas en estudios en los que no estaban involucradas plantas leguminosas. Por ejemplo, Filella & Peñuelas (2003b) demostraron la repartición de fuentes de N entre especies coexistentes en un ecosistema

Mediterráneo basándose en medidas de $\delta^{15}N$ foliar. Las distintas fuentes de nitrógeno (como nitrato o amonio) difieren en su $\delta^{15}N$ como resultado de los procesos de mineralización y nitrificación (Högberg 1997). Además, el $\delta^{15}N$ del suelo tiende a mostrar valores más enriquecidos con la profundidad en el perfil edáfico (Natelhoffer & Fry 1988, Peñuelas *et al.* 2011).

Otros estudios realizados a varias escalas (local, regional y global) han encontrado que los valores de $\delta^{15}N$ foliar se relacionan con la concentración de N foliar en especies vegetales no-fijadoras de N_2 atmosférico (Bai *et al.* 2009, BassiriRad *et al.* 2003, Craine *et al.* 2009, Hobbie, Macko & Williams 2000), evidenciando de este modo la relación existente entre los valores de $\delta^{15}N$ del material vegetal y la demanda y disponibilidad de nutrientes para la planta.

Utilidad de los métodos isotópicos en estudios de ecofisiología vegetal

El número de los estudios de ecofisiología vegetal que emplean métodos basados en la abundancia natural de los isótopos estables ha crecido de forma exponencial en los últimos años, poniendo en evidencia la utilidad esta técnica.

Algunos estudios han utilizado con éxito las técnicas de isótopos estables para demostrar la repartición de los recursos del suelo entre especies vegetales coexistentes dentro de una misma comunidad. El análisis de la composición isotópica del agua del xilema ha ayudado a identificar las fuentes de agua utilizadas por distintas especies vegetales (p.ej. Dawson & Ehleringer 1991, Ehleringer & Dawson 1992, Filella & Peñuelas 2003b, Querejeta *et al.* 2007, Stratton *et al.* 2000). En ecosistemas áridos y semiáridos se ha demostrado la segregación espacial y temporal en la utilización del agua por parte de especies coexistentes que muestran diferencias en su habilidad para explotar el agua del suelo (Ehleringer *et al.* 1991, Williams & Ehleringer 2000). Esto implica la existencia de una repartición de los recursos entre plantas que coexisten en comunidades vegetales desarrolladas en ambientes fuertemente limitados por el agua. Otros estudios basados en métodos isotópicos también han encontrado que las plantas coexistentes utilizan diferentes reservorios (pools) de nutrientes. En concreto, el análisis de la composición isotópica foliar de nitrógeno ha permitido identificar la repartición de fuentes de N a nivel inter-específico (Bustamante *et al.* 2004, Filella & Peñuelas 2003b, Ometto *et al.* 2006). Pero los isótopos estables no sólo han ayudado a asociar diferentes

fuentes de recursos a cada planta, sino que también han permitido identificar las distintas estrategias de utilización de los recursos por parte de las plantas (como son las variaciones espaciales y temporales en el uso de esos recursos). En este sentido, la composición isotópica en carbono del material vegetal ha permitido distinguir entre estrategias más conservadoras y más derrochadoras en el uso de los recursos (Escudero, Mediavilla & Heilmeier 2008, Saura-Mas & Lloret 2010).

Los métodos isotópicos también se han utilizado con éxito para identificar relaciones de competencia o facilitación inter- o intra-específicas. Algunos estudios han encontrado relaciones de competencia por el agua y/o nutrientes a nivel inter-específico (Grams *et al.* 2007, Grams & Matyssek 2010, Leffler & Caldwell 2005, Williams, Richards & Caldwell 1991) e intra-específico (Ramírez *et al.* 2009, Sala *et al.* 2005). Junto a estos trabajos, otros autores han estudiado los procesos ecofisiológicos relacionados con la invasión de especies vegetales (Hellmann *et al.* 2011, Rascher *et al.* 2011) y al parasitismo (Cernusak, Pate & Farquhar 2004). En cambio, otros estudios han encontrado relaciones de facilitación entre plantas. Entre ellos cabe destacar los estudios centrados en la redistribución hidráulica por las raíces de las plantas (p.ej. Dawson 1993, Filella & Peñuelas 2003a). Sin embargo, los procesos de redistribución hidráulica no siempre resultan en interacciones de facilitación entre plantas, como demostraron (Armas *et al.* 2010) con la redistribución hidráulica de agua salina por parte de *Pistacia lentiscus* que afecta negativamente a *Juniperus phoenicea* en ecosistemas dunares.

El análisis de la composición isotópica de los anillos de crecimiento permite un estudio retrospectivo a largo plazo del estado fisiológico de las plantas y en algunos casos han servido por ejemplo para estimar las diferencias inter-específicas en la resistencia de las plantas a la sequía (Battipaglia *et al.* 2009) o en sus estrategias de uso del agua (Gebrekirstos *et al.* 2011) o para evaluar el efecto a largo plazo de las relaciones de competencia entre plantas (Linares *et al.* 2009). También se han empleado para evaluar el efecto a largo plazo del aclareo en el estatus fisiológico de los árboles remanentes una vez atenuada la intensidad de la competencia por los recursos del suelo (Brooks & Mitchell 2011, Martín-Benito *et al.* 2010, McDowell *et al.* 2003).

Por todo ello, las técnicas de isótopos estables son una herramienta útil que se utilizará en esta Tesis Doctoral para estudiar las relaciones hídricas de las plantas.

OBJETIVOS DE LA TESIS DOCTORAL

Los modelos de cambio climático predicen para el SE ibérico un descenso de la precipitación con una tendencia hacia la presencia periodos secos cada vez más largos (Machado *et al.* 2011). Junto con un aumento de las temperaturas esto producirá una intensificación de la aridez y del estrés hídrico de las plantas. Ante este escenario, es importante conocer las estrategias de utilización del agua por las plantas y cómo el recurso más limitante se reparte entre las distintas especies que componen las comunidades vegetales del SE ibérico. Conociendo la estrategia de las distintas especies podríamos predecir la respuesta de las comunidades vegetales ante el cambio climático u otros factores externos que alteren la disponibilidad de recursos para las plantas. Esto ayudaría también en la toma de decisiones sobre las actuaciones de manejo forestal que son más apropiadas.

Como consecuencia de las políticas de restauración hidrológico-forestal del siglo XX, vastas áreas del SE ibérico están ocupadas por repoblaciones forestales de *Pinus halepensis* Mill. (Cortina *et al.* 2011). En la actualidad estas repoblaciones han dado lugar a masas forestal monoespecíficas y densas en las que el crecimiento de los pinos es bajo y además han fracasado a la hora de favorecer el establecimiento de nuevas especies para evolucionar hacia comunidades vegetales más naturalizadas (Maestre *et al.* 2003). Es preciso conocer los procesos ecofisiológicos que se dan en estas comunidades vegetales para ayudar a decidir las actuaciones forestales más adecuadas que permitan mejorar la resistencia de estas comunidades frente al cambio climático y ayudar en su transición a comunidades vegetales maduras de etapas sucesionales más avanzadas.

El objetivo general de la presente Tesis Doctoral es el de estudiar las estrategias de uso del agua de algunas de las especies de plantas más comunes que crecen de forma natural en comunidades vegetales del SE ibérico semiárido para evaluar cómo estas especies se reparten el recurso más limitante en esta zona, que es el agua. Así mismo, se pretende estudiar el tipo de interacciones que se dan entre plantas dentro de las repoblaciones de *P. halepensis* y cómo éstas afectan al uso de los recursos por parte de las plantas. Por último, se pretende evaluar la utilidad de las técnicas de isótopos estables para el estudio ecofisiológico de la repartición de los recursos y las interacciones entre plantas en comunidades vegetales del SE ibérico semiárido.

La hipótesis de partida de esta memoria de Tesis Doctoral es que los pinares y matorrales naturales de etapas sucesionales avanzadas muestran una alta diversidad funcional, ya que en ellos coexisten especies vegetales con estrategias complementarias en la utilización del recurso agua. Por el contrario, las interacciones de competencia por el agua entre plantas vecinas prevalecen dentro de las repoblaciones monoespecíficas de *P. halepensis*, de tal modo que el agua se torna un recurso aún más limitante para las plantas en este tipo de comunidades artificiales.

Los objetivos concretos de la Tesis Doctoral son:

- Caracterizar la estrategia de utilización del agua de algunas de las especies vegetales más comunes en comunidades vegetales naturales y de repoblación del SE ibérico semiárido, mediante el empleo sistemático de técnicas de isótopos estables.
- Evaluar el efecto de una de las técnicas de manejo silvícola más utilizada en repoblación forestal (el aclareo) sobre el patrón de uso del agua y estatus hídrico de la vegetación implantada.
- Comparar la dinámica de utilización del agua por parte de la vegetación en comunidades vegetales naturales y en pinares de repoblación vecinos, identificando en ambos casos posibles procesos de competencia y facilitación a nivel intra- e inter-específico.

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CAPÍTULO 2

ISOTOPES REVEAL CONTRASTING WATER USE STRATEGIES AMONG COEXISTING PLANT SPECIES IN A MEDITERRANEAN ECOSYSTEM

Isotopes reveal contrasting water use strategies among coexisting plant species in a Mediterranean ecosystem

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ABSTRACT

Variation in the stable carbon and oxygen isotope composition ($\delta^{13}C$, $\Delta^{18}O$) of co-occurring plant species may reflect the functional diversity of water-use strategies present in natural plant communities.

We investigated patterns of water use among ten coexisting plant species representing diverse taxonomic groups and life-forms in semiarid SE Spain, by measuring their leaf $\delta^{13}C$ and $\Delta^{18}O$, the oxygen isotope ratio of stem water, and leaf gas exchange rates.

Across species, $\Delta^{18}O$ was tightly negatively correlated with stomatal conductance (g_s), whereas $\delta^{13}C$ was positively correlated with intrinsic water use efficiency (WUE_i). Broad inter-specific variation in $\Delta^{18}O$, $\delta^{13}C$ and WUE_i was largely determined by differences in g_s, as indicated by a strong positive correlation between leaf $\delta^{13}C$ and $\Delta^{18}O$ across species

The ten co-occurring species segregated along a continuous ecophysiological gradient defined by their leaf $\delta^{13}C$ and $\Delta^{18}O$, thus revealing a wide spectrum of stomatal regulation intensity and contrasting water use strategies ranging from "profligate/opportunistic" (high g_s, low WUE_i) to "conservative" (low g_s, high WUE_i). Coexisting species maintained their relative isotopic rankings in two years with contrasting rainfall, which suggests the existence of species-specific "isotopic niches" that reflect ecophysiological niche segregation in dryland plant communities.

Key words: dual isotope approach, stomatal conductance, water use efficiency, water uptake, drought, isotopic niches, functional diversity, coexistence.

INTRODUCTION

Environmental conditions and resource supply rates in water-limited habitats are highly heterogeneous in space and time, thus providing axes of ecological differentiation that can facilitate species coexistence (Chesson *et al.* 2004). Coexisting plant species often adopt contrasting resource use strategies through differences in key traits such as rooting depth or the phenology of resource use, which leads to spatial and temporal partitioning of limiting resources like water (Orians & Solbrig 1977, Peñuelas, Terradas & Lloret 2011, Schwinning & Ehleringer 2001). In water-limited ecosystems with pulsed resources availability, one important mechanism that

can influence the structuring of plant communities is hydrological niche segregation among co-occurring species (Dawson 1990, Silvertown *et al.* 1999). Contrasting, but equally successful strategies for coping with water stress can coexist in semiarid plant communities (Jacobsen *et al.* 2008, West *et al.* 2012), even if there are certain ecophysiological constraints that limit functional diversity and determine inevitable tradeoffs among plant traits. For example, a trade-off exists between the resource consumption and growth rates of plant species during resource pulses, and their ability to withstand long inter-pulse intervals or chronically low resource availability (Angert *et al.* 2009, Chesson *et al.* 2004).

Plant species with contrasting water use strategies are known to coexist in Mediterranean ecosystems (Peñuelas *et al.* 2011, West *et al.* 2012). The responses of plant species to their fluctuating environment are strongly influenced by their water use strategies (Xu & Li 2006). Relevant traits that determine water use strategies are acquisition efficiency (root traits) and use efficiency (leaf and stem traits). Ecophysiological attributes related to plant water use strategies may be inherent traits of species that can help group them into functional types. For example, Herrera (1984) identified a range of plant functional types showing contrasting water-relations traits in Mediterranean ecosystems, with evergreen sclerophyllous species vs. drought deciduous or semi-deciduous species at the opposite extremes of a continuous ecophysiological gradient.

Leaf stomatal conductance and water use efficiency are key ecophysiological attributes of plant species that have seldom been considered in formulations of plant functional typologies at the global scale. For example, the widely used worldwide leaf economics spectrum (Wright *et al.* 2004) instead emphasizes the importance of other key leaf traits (leaf mass area, photosynthetic assimilation rate, leaf nitrogen and phosphorus concentrations, dark respiration rate and leaf lifespan). Identifying a continuous spectrum of stomatal regulation intensity (i.e. tight vs. loose stomatal control of leaf gas exchange) and/or water use efficiency across multiple plant taxa and life forms would help to fill this gap in current formulations of plant functional diversity, and would improve our understanding of the mechanisms of species coexistence in water limited habitats.

Analyzing the stable carbon and oxygen isotope ratios of co-occurring plant species exposed to the same environmental conditions can be useful to characterize the diversity of functional traits related to water relations in natural plant communities (Angert *et al.* 2009, Escudero, Mediavilla & Heilmeier 2008, Filella & Peñuelas 2003, Saura-Mas & Lloret 2010, Werner & Máguas 2010). Measuring the oxygen isotope enrichment of plant material above source water ($\Delta^{18}O = \delta^{18}O_{plant} - \delta^{18}O_{source\ water}$) in coexisting plant species can help identify inter-specific differences in stomatal conductance (g_s), as $\Delta^{18}O$ is inversely related to the ratio of atmospheric to leaf intercellular water vapour pressure (e_a/e_i), and can thus provide a time-integrated indication of g_s and transpiration during the growing season (Barbour 2007, Farquhar, Cernusak & Barnes 2007). The carbon stable isotope composition ($\delta^{13}C$) of leaves in C_3 plants depends on the ratio between the partial pressures of CO_2 at the sites of carboxylation in the chloroplast and in the ambient air (c_i/c_a), and is a good proxy of leaf-level intrinsic water use efficiency (WUE_i), which is given by the ratio between leaf net photosynthetic rate (A) and g_s (Dawson *et al.* 2002, Farquhar, Ehleringer & Hubick 1989). Since both stomatal conductance and photosynthetic rates have an influence on c_i/c_a, it is often difficult to determine the extent to which inter-specific differences in plant $\delta^{13}C$ are determined by differences in g_s, A, or both (e.g. Keitel *et al.* 2006). The oxygen stable isotope composition of plant tissue ($\delta^{18}O$) can help separate the independent effects of A and g_s on $\delta^{13}C$, because $\delta^{18}O$ shares dependence on g_s with $\delta^{13}C$, but is thought to be independent of variation in A (Grams *et al.* 2007, Moreno-Gutiérrez *et al.* 2011, Roden & Farquhar 2012, Scheidegger *et al.* 2000). On the other hand, the hydrogen and oxygen stable isotope composition of water (δD and

$\delta^{18}O$, respectively) extracted from plant stems accurately reflects the isotope ratio of soil water used by plants, as no isotopic fractionation occurs during soil water uptake by roots (Barbour 2007).

The aim of this work was to characterize the water use strategies of ten coexisting plant species of widely contrasting phylogenies and life forms in a semiarid Mediterranean ecosystem, using a dual C and O stable isotope approach. As noted by Wright *et al.* (2004), much of the total variation along the universal leaf functional-trait spectrum occurs among coexisting plant species, and we expected the same to be true for both leaf traits related to water relations and leaf isotope ratios. We measured the stable isotope composition of leaves and stem water in adult individuals in order to assess the usefulness of using plant stable C and O isotope ratios as indicators of species' water use strategies under field conditions. Previous investigations have urged caution about simplistic interpretations of water use efficiency (WUE) based on inter-specific variation in $\delta^{13}C$ when comparing plant species with wide-ranging leaf anatomy/physiology (e.g. Seibt *et al.* 2008, Warren & Adams 2006). However, we still feel that there is power in using $\delta^{13}C$ as a "relative index" of WUE for ranking species occupying the same environment (see Dawson & Ehleringer 1993), and therefore utility in applying the dual C and O isotope approach for investigating plant water use strategies, particularly when applied in a strongly water-limited ecosystem. As such, we hypothesized that there would be large differences in stomatal conductance, water use efficiency, depth of water uptake and overall water use pattern among our ten target plant species during peak growing season, and that these differences would be reflected in their C and O isotope ratios.

MATERIALS AND METHODS

Study site and sampling

The study was conducted near the city of Murcia (SE Spain), in an open woodland community with widely scattered *Pinus halepensis* Mill. trees, and an understory dominated by *Stipa tenacissima* L., *Rosmarinus officinalis* L. and *Anthyllis cytisoides* L. The terrain in the experimental area is hilly (140-170m asl., < 20 % slopes), and the soils are haplic calcisols and lithic leptosols (according to FAO classification). The climate is semiarid Mediterranean, with mean annual precipitation of 288 mm and an average annual temperature of 19ºC.

In May 2007 and in April 2010, fully sun-exposed leaves and twig sections (approximately 10 mm in diameter and 20 mm in length) were collected from adult individuals of ten coexisting species of widely contrasting taxonomy and life form (see Table 1). In May 2007, soil samples

Table 1. Brief description of the 10 coexisting plant species included in this study.

Species	Plant family	Lineage age	Life form	Leaf habit
Pistacia lentiscus L.	Anacardiaceae	PM	Large shrub	E
Olea europaea L.	Oleaceae	PM	Large shub/ tree	E
Chamaerops humilis L.	Arecaceae	PM	Shrubby palm	E
Pinus halepensis Mill.	Pinaceae	PM	Tree	E
Nerium oleander L.	Apocynaceae	PM	Large shrub	E
Anthyllis cytisoides L.	Fabaceae	M	Small shrub	D
Rhamnus lycioides L.	Rhamnaceae	PM	Medium shrub	SD
Rosmarinus officinalis L.	Lamiaceae	M	Small shrub	SD
Stipa tenacissima L.	Poaceae	-	Tussock grass	E
Helianthemum syriacum (Jacq.) Dum. Cours.	Cistaceae	M	Dwarf shrub	SD

Linage age: PM = Pre-Mediterranean, M = Mediterranean. Leaf habit: E = evergreen, D = drought deciduous, SD = drought semi-deciduous.

were collected at depth intervals of 10 cm with an auger probe, from 7 different locations. After collection, lignified twig sections and soil samples were immediately placed in capped vials, wrapped with Parafilm, and stored in the freezer until water extraction using a cryogenic vacuum distillation line (Ehleringer, Roden & Dawson 2000). During each sampling campaign, plant height and crown diameter (measured at two directions) were recorded (except for the pines) and plant biovolume was estimated as the volume of the upper-half ellipsoid with those height and diameters. During 2008, rainfall water samples from discrete rainfall events were collected with a pluviometer (9 cm in diameter) placed at 1m height in an open area. Mineral oil was used to prevent evaporation of rainwater samples in the pluviometer. Rainwater samples were taken within 1-2 days after each rainfall event.

Isotope composition of leaves

Bulk leaf samples were oven-dried at 60 ºC and finely ground with a ball mill. Leaf α-cellulose was extracted from samples of April 2010 using the method described by Leavitt & Danzer (1993) with the addition of a third step of purification with a 17% w/v NaOH solution, followed by an acetic acid solution (10%) and thorough rinsing with double distilled water.

The carbon isotope ratio of leaf material ($\delta^{13}C$) was analysed using elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS). The oxygen isotope ratio of leaf material ($\delta^{18}O$) was determined with a Finnigan MAT Delta Plus XL IRMS (Finnigan MAT, Bremen, Germany) following the method described in Farquhar, Henry & Styles (1997). This method uses a high purity alumina pyrolysis tube, a pyrolysis temperature of 1130ºC, chloropentane doping of the carrier gas, and a Porapak Q GC column before the molecular sieve column. Isotope analyses were conducted at the Center for Stable Isotope Biogeochemistry, University of California-Berkeley (USA). Isotope ratios are expressed in delta notation (‰) relative to an accepted reference standard: V-PDB for carbon isotope values and V-SMOW for oxygen isotope values. Long-term (6+ year) external precisions for carbon and oxygen isotope analyses are 0.14 and 0.23‰, respectively.

Isotope composition of rainfall, soil and stem waters

Isotopic analyses of rainfall, soil and stem water samples were conducted at the Center for Stable Isotope Biogeochemistry, University of California-Berkeley (USA). For $\delta^{18}O$ analyses, 0.2ml of water samples was equilibrated with an atmosphere of 0.2% of CO_2 for 48h at room temperature (21-23ºC). The $\delta^{18}O$ of equilibrated samples was measured using a continuous flow isotope ratio mass spectrometer (Finnigan MAT Delta Plus XL; ThermoFinnigan, Bremen, Germany) connected to a GasBench II interface (GB, ThermoFinnigan). δD of water samples was analysed using a chromium reduction system at 900ºC (Finnigan MAT H/Device) coupled to a Finnigan MAT Delta Plus XL mass spectrometer (ThermoFinnigan MAT, Bremen, Germany). The long-term external precision for oxygen and hydrogen isotope analyses was 0.12‰ and 0.8‰, respectively. $\delta^{18}O$ and δD values are expressed in delta notation (‰) relative to the international standard V-SMOW.

The oxygen isotopic composition of leaf tissue is reported both as bulk leaf $\delta^{18}O$ and as oxygen isotope enrichment above that of source water ($\Delta^{18}O$). The latter was calculated as follows:

$$\Delta^{18}O = \delta^{18}O_{leaf} - \delta^{18}O_{stem\ water}$$

The Local Meteoric Water Line was calculated by linear regression of the $\delta^{18}O$ and δD values of rainwater samples. We used stem water $\delta^{18}O$ values (rather than δD) as a measure of inter-specific differences in source water, because xerophytic plants might possess the capacity to discriminate against deuterium isotopes during soil water uptake by roots (Ellsworth & Williams 2007).

Leaf gas exchange measurements

Net photosynthetic rate (A) and stomatal conductance (g_s) during peak growing season (April 2010) were measured in the ten coexisting species, using a portable photosynthesis system (LI-6400, LICOR, Inc., Lincoln, NE, USA) equipped with a LI-6400-40 Leaf Chamber Fluorometer and a LICOR 6400-01 CO_2 injector. Leaf gas exchange was measured on 1-year-old, fully sun-exposed leaves that were placed in a 2 cm^2 leaf cuvette. The CO_2 concentration in the cuvette was maintained at 380 µmol mol^{-1} CO_2. Measurements were done at saturating light of 1800 µmol m^{-2} s^{-1}, and at ambient air temperature and relative humidity. The leaf-to-air water vapour concentration gradient ranged between 0.75 and 1.2 mmol mol^{-1} for all measurements, and the air flow was set to 350 µmol s^{-1}. All leaf gas exchange measurements were conducted at mid-morning between 9:00-11:00 h (local standard time; 7:00-9:00 GMT) on sunny days and were expressed on a total leaf surface area basis. The total surface area of pine needles enclosed within the leaf cuvette was measured by using an A3 flatbed scanner (HP Deskcan) and the WinRhizo software (Regent Instruments Inc., Québec, Canada). Total needle surface area values measured by this method were on average 7.5% higher (2.15 cm^2) than the area of the leaf cuvette (2.00 cm^2). Intrinsic water-use efficiency (WUE_i) was calculated as A/g_s.

Statistical analyses

All statistical analyses were performed with SPSS software (version 17.0, SPSS Inc., Chicago). The relationships between analyzed variables were assessed across species with simple linear regressions.

RESULTS

Leaf isotope ratios and gas exchange rates

Our ten target species showed a large range of stomatal conductance (from 0.069 to 0.175 mol m^{-2} s^{-1}; Fig. 1a) and intrinsic water use efficiency values (36.1-149.7 µmol CO_2/mol H_2O; Fig 1b) during peak growing season (April 2010). There was a remarkably strong correlation between the oxygen isotope composition of leaf cellulose and leaf stomatal conductance across species: g_s was tightly and negatively associated with leaf

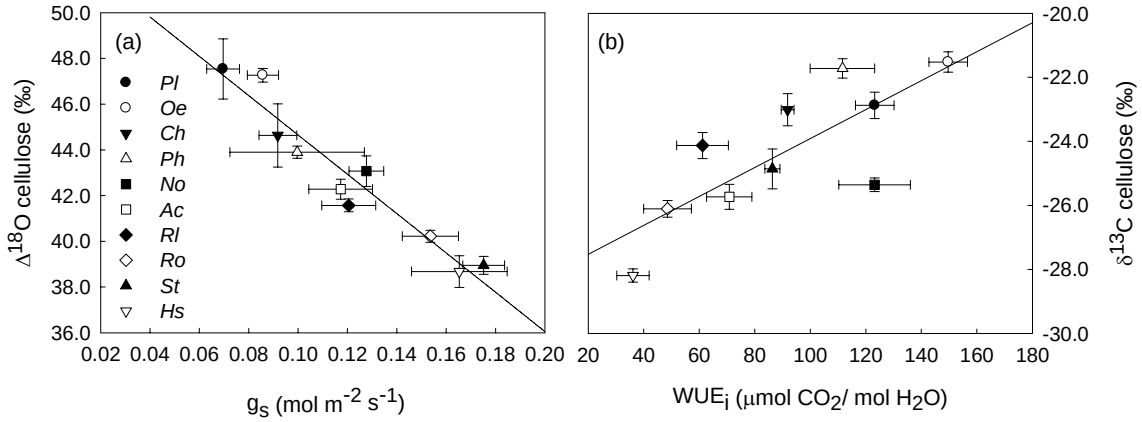

Figure 1. Cross-species (n = 10; April 2010) relationships between: (a) leaf cellulose oxygen isotope enrichment above source water ($\Delta^{18}O_{cellulose}$) vs. stomatal conductance (g_s), $r^2 = 0.92$, $P < 0.001$; (b) leaf cellulose carbon isotope ratio ($\delta^{13}C_{cellulose}$) vs. intrinsic water use efficiency (WUE_i), $r^2 = 0.62$, $P = 0.007$. Error bars represent 1SE. *Pl = Pistacia lentiscus* (n = 3), *Oe = Olea europaea* (n = 3), *Ch = Chamaerops humilis* (n = 3), *Ph = Pinus halepensis* (n = 3), *No = Nerium oleander* (n = 4), *Ac = Anthyllis cytisoides* (n = 9), *Rl = Rhamnus lycioides* (n = 5), *Ro = Rosmarinus officinalis* (n = 3), *St = Stipa tenacissima* (n = 3), *Hs = Helianthemum syriacum*(n = 4).

Table 2. Mean leaf nitrogen concentrations in May 2007 and April 2010 (± SE) and photosynthetic rate in April 2010 (A; ± SE) of the 10 target species.

SPECIES	N (%) in 2007	N (%) in 2010	A (μmol m^{-2} s^{-1})
Pistacia lentiscus L.	1.41 ± 0.06	1.32 ± 0.06	8.37 ± 0.35
Olea europaea L.	1.60 ± 0.06	1.51 ± 0.09	12.59 ± 0.52
Chamaerops humilis L.	1.43 ± 0.09	1.36 ± 0.06	8.40 ± 0.25
Pinus halepensis Mill.	1.12 ± 0.04	1.34 ± 0.05	10.51 ± 1.90
Nerium oleander L.	1.26 ± 0.10	1.42 ± 0.02	15.32 ± 0.80
Anthyllis cytisoides L.	1.87 ± 0.13	1.62 ± 0.12	7.94 ± 0.98
Rhamnus lycioides L.	2.02 ± 0.08	2.36 ± 0.09	7.75 ± 0.62
Rosmarinus officinalis L.	1.16 ± 0.06	1.43 ± 0.02	7.50 ± 1.48
Stipa tenacissima L.	0.98 ± 0.06	0.94 ± 0.11	15.16 ± 0.98
Helianthemum syriacum (Jacq.) Dum. Cours.	1.07 ± 0.01	1.82 ± 0.08	5.80 ± 0.95

cellulose $\Delta^{18}O$ (Fig. 1a) and $\delta^{18}O$ ($r^2 = 0.85$, $P < 0.001$). Weaker associations were found between g_s and bulk leaf $\Delta^{18}O$ ($r^2 = 0.43$, $P = 0.039$) and $\delta^{18}O$ ($r^2 = 0.32$, $P = 0.087$). Intrinsic water use efficiency (WUE_i) was positively associated with leaf cellulose $\delta^{13}C$ (Fig. 1b) and bulk leaf $\delta^{13}C$ ($r^2 = 0.55$, $P = 0.014$) across species. On the contrary, leaf cellulose $\delta^{13}C$ and $\Delta^{18}O$ were not correlated to variations in photosynthetic rate (see Table 2; $P = 0.399$ and $P = 0.755$, respectively) or leaf nitrogen concentration (Table 2; $P = 0.538$ and $P = 0.723$, respectively) across species.

Cellulose and bulk leaf $\delta^{13}C$ correlated well across species ($r^2 = 0.86$, $P < 0.001$, $n = 10$), whereas the correlation between cellulose and bulk leaf $\delta^{18}O$ was significant but weaker ($r^2 = 0.45$, $P = 0.033$, $n = 10$).

Leaf cellulose $\delta^{13}C$ and $\Delta^{18}O$ were strongly and positively associated across species (Fig. 2). Bulk leaf $\delta^{13}C$ and $\delta^{18}O$ were positively correlated as well in both 2007 ($r^2 = 0.81$, $P < 0.001$) and 2010 ($r^2 = 0.67$, $P = 0.004$). The positive correlation between bulk leaf $\delta^{13}C$ and $\Delta^{18}O$ showed rather similar slopes in both May 2007 ($r^2 = 0.77$, $P = 0.001$, $\delta^{13}C = 0.72\Delta^{18}O - 52.03$) and April 2010 ($r^2 = 0.75$, $P = 0.001$, $\delta^{13}C = 0.66\Delta^{18}O - 50.65$). We also found strong relationships between g_s and $\delta^{13}C$ of leaf cellulose (negative, $r^2 = 0.62$, $P = 0.007$), and between WUE_i and leaf cellulose $\Delta^{18}O$ (positive, $r^2 = 0.68$, $P = 0.003$) across species.

Species' bulk leaf $\Delta^{18}O$ values were tightly correlated between years with contrasting climatic conditions (2007 vs. 2010; Fig. 3). Species' bulk leaf $\delta^{18}O$ and $\delta^{13}C$ values were correlated between years as well (2007 vs. 2010; $r^2 = 0.78$, $P=0.001$ and $r^2 = 0.68$, $P= 0.003$, respectively), even though leaf $\delta^{18}O$ values were consistently more enriched in 2007 than in 2010 (by 0.8-3.4‰, depending on the species) due to more xeric climate conditions in the former year.

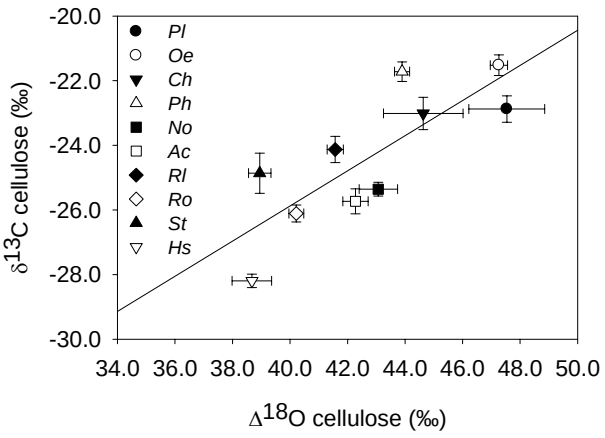

Figure 2. Relationship between carbon isotope ratios ($\delta^{13}C_{cellulose}$) and oxygen isotope enrichment above source water ($\Delta^{18}O_{cellulose}$) of leaf cellulose across species in April 2010 ($r^2 = 0.65$, $P = 0.005$, $n = 10$). Error bars represent 1SE. Symbols as in Fig. 1

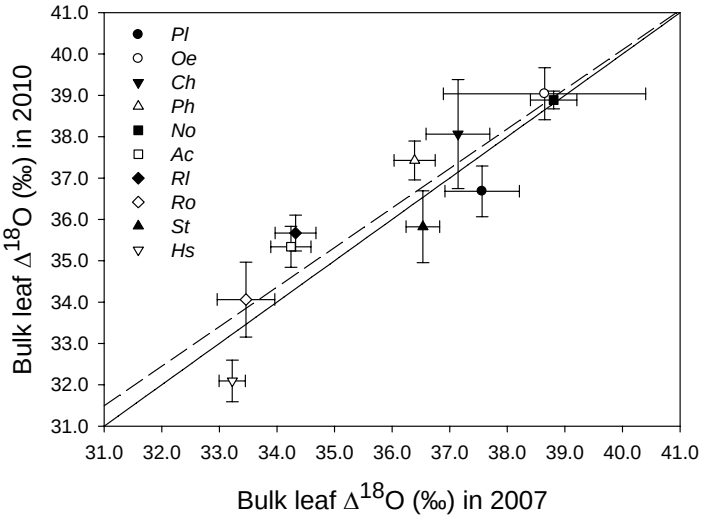

Figure 3. Leaf oxygen isotope enrichment above source water (bulk leaf $\Delta^{18}O$) of 1 coexisting plant species in two years with contrasting climatic conditions (2007 vs. 2008; $r^2 = 0.83$, $P < 0.001$, $n = 10$, $\Delta^{18}O_{2010} = 0.96\Delta^{18}O_{2007} + 1.83$). Error bars represent one SE. The dotted line represents the linear regression; the solid line represents the 1:1 relationship. Symbols as in Fig. 1

Bulk leaf $\delta^{13}C$ values were also more enriched in 2007 than in 2010 for 8 out of 10 species, with differences ranging from 0.1 to 2.1‰.

Isotope composition of soil and stem water

Across species, stem water $\delta^{18}O$ values were consistently more depleted (by 0.9-3.5‰ depending on the species) during a cool and wet period in April 2010 (rainfall was 46.8mm and mean temperature was 12ºC during the 30 days prior to sampling) than during a warm and dry period in May 2007 (rainfall was only 18.3mm and mean temperature was 21ºC during the 30 days prior to sampling).

Soil water $\delta^{18}O$ in the 0-40 cm depth interval showed a steep vertical gradient in May 2007 (Fig 4 inset), and mean stem water $\delta^{18}O$ (which reflects the isotopic signal of source water used by plants) differed markedly among coexisting species. The stem water $\delta^{18}O$ values of *H. syriacum*, *S. tenacissima*, *A. cytisoides* and *R. officinalis* (ranging from -3.5 ± 0.4‰ to -4.1 ± 0.2‰) were similar to the $\delta^{18}O$ values of water in the upper soil layers (< 40 cm depth; see inset in Fig.4) at this time. By contrast, the remaining species showed more depleted stem water $\delta^{18}O$ values (from -4.6 ± 0.3‰ to -5.9 ± 0.2‰) which were more negative than the $\delta^{18}O$ values of water in the upper soil layers (see inset in Fig.4).

In sharp contrast to the pattern observed during a warm and dry period in May 2007, the co-occurring species showed largely overlapping stem water $\delta^{18}O$ values during a cool and wet period in April 2010, with the only exception being *H. syriacum* (which again exhibited the highest mean value of all the target species, -5.7 ± 0.4‰). All the other species showed rather similar stem water $\delta^{18}O$ values (ranging from -6.5 ± 0.4‰ to -7.5 ± 0.3‰) at this time.

Across species, stem water $\delta^{18}O$ in May 2007 was negatively related to leaf cellulose $\delta^{18}O$ ($r^2 = 0.71$, $P = 0.002$) and $\delta^{13}C$ ($r^2 = 0.41$, $P = 0.045$) in April 2010. Stem water $\delta^{18}O$ in May 2007 was also significantly related to stomatal conductance (positively, $r^2 = 0.60$, $P = 0.008$) and WUE_i (negatively, $r^2 = 0.65$, $P = 0.005$) during peak growing season in April 2010.

Across species, stem water $\delta^{18}O$ was negatively associated with mean plant size (biovolume; $r^2 = 0.86$, $P < 0.001$, $n = 9$) in May 2007, thus indicating that larger species were better capable of extracting isotopically depleted water from deeper soil layers during a dry period. However *P. halepensis*, which is a conifer tree with a dimorphic (shallow and deep) root system, deviated from this pattern. Plant size was positively associated with leaf cellulose $\Delta^{18}O$ ($r^2 = 0.63$, $P = 0.011$, $n = 9$) and $\delta^{13}C$ ($r^2 = 0.64$, $P = 0.010$, $n = 9$) across species when *P. halepensis* was excluded from these correlations. Moreover,

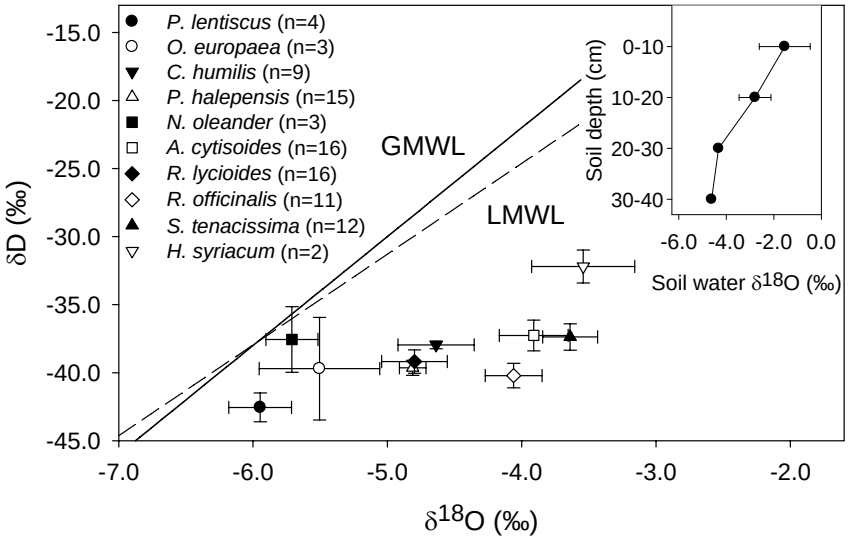

Figure 4. Average deuterium and oxygen isotope ratios (δD and $\delta^{18}O$, respectively) of stem water of the target plant species in May 2007 against the Global Meteoric Water Line (GMWL, straight line) and the Local Meteoric Water Line (LMWL, dotted line). Inset figure: changes in soil water $\delta^{18}O$ with soil depth in May 2007. Error bars represent one SE. The LMWL is described by the equation $\delta D = 6.67\delta^{18}O + 2.06$ (Fig. 1a). Mean deuterium excess (*d*-excess) of local precipitation was 9.32 ± 1.15‰.

plant size was negatively correlated with stomatal conductance ($r^2 = 0.56$, P = 0.021, n=9) and positively correlated with WUE_i ($r^2 = 0.52$, P = 0.027, n=9) across species.

DISCUSSION

Dual Isotope Findings

We found that leaf $\Delta^{18}O$ and $\delta^{13}C$ were reliable indicators of stomatal conductance (g_s) and intrinsic water use efficiency (WUE_i), respectively, across ten co-occurring species of contrasting life forms and phylogenetically distant plant families (Table 1, Fig. 1). There was a remarkably strong correlation between the oxygen isotope composition of leaf cellulose and leaf stomatal conductance across species (Fig. 1a). Our data indicate that analysing the stable isotope composition of leaf cellulose is more appropriate when performing comparisons across species (particularly for $\delta^{18}O$), because bulk leaf material contains species-specific proportions of organic compounds (e.g. tannins, resins, waxes, oils, etc.), with different isotopic ratios that add noise to the relationship between bulk leaf $\delta^{13}C$, $\delta^{18}O$ and leaf gas exchange parameters.

Leaf cellulose $\delta^{13}C$ and $\Delta^{18}O$ were strongly positively associated across our 10 target species (Fig. 2), which suggests tight stomatal control of the physiology that imparts leaf-level isotope composition (discrimination and isotope routing into cellulose; Barbour 2007). We take this as evidence that the ten co-existing species we studied are segregated along a "functional continuum" defined by intrinsic ecophysiological differences that regulate both leaf-level C and O isotope ratios. The large inter-specific differences in WUE_i, $\Delta^{18}O$ and $\delta^{13}C$ were unrelated to the similarly large variation in photosynthetic rate among them (Table 2). Inter-specific variation in $\delta^{13}C$ reflected real and large differences in degree of stomatal regulation of leaf-level WUE_i among co-occurring species in this semiarid ecosystem (Cernusak *et al.* 2007, Cernusak *et al.* 2008), rather than (or in addition to) inter-specific differences in mesophyll conductance to CO_2, biochemical photosynthetic capacity or other leaf traits known to also influence plant $\delta^{13}C$ (Grassi &

Magnani 2005, Seibt *et al.* 2008). This said, we cannot rule out the potential that concurrent changes in mesophyll conductance to CO_2 and leaf hydraulic conductance (Ferrio *et al.* 2012) could have also contributed to the strong positive association found between $\Delta^{18}O$ and $\delta^{13}C$ across species. In this regard, additional research is needed.

Species in this semiarid ecosystem appear to operate at relatively constant A/g_s (photosynthetic rate vs. stomatal conductance) set points, which appears to dictate their relative isotope rankings through time (Ehleringer & Cerling 1995). The high year to year stability of C and O isotope rankings among coexisting plant species (Ehleringer & Cerling 1995; Fig.3) suggests the existence of distinct species "isotopic niches" that offer insight into the diversity of functional (ecophysiological) niches related to water use patterns.

Few studies have examined the relationship between plant isotope ratios and leaf gas exchange rates across plant species growing under similar environmental conditions. In contrast to our results, Kahmen *et al.* (2008) found that inter-specific variation in leaf water $\delta^{18}O$ (the major determinant of leaf $\delta^{18}O$) across 17 *Eucalyptus* species growing in a common garden experiment was not related to differences in leaf transpiration. In another common garden experiment, Cernusak *et al.* (2008) found that whole-plant ^{13}C discrimination in tropical tree seedlings was related to transpiration efficiency across species, which was in turn largely controlled by stomatal conductance. Wang *et al.* (1998) measured leaf cellulose $\delta^{13}C$ and $\delta^{18}O$ in 90 plant species grown under the same climatic conditions in the Jerusalem Botanical Garden, and found a significant (but weak) positive correlation between leaf $\delta^{13}C$ and $\delta^{18}O$ across species. In that study, transpiration was negatively correlated with leaf cellulose $\delta^{13}C$ and marginally negatively correlated with leaf cellulose $\delta^{18}O$ across species (Wang et al., 1998). Here we report similar (but stronger) relationships, which suggests that plant species C and O isotope ratios may better reflect species water use patterns in strongly water-limited ecosystems (were stomatal conductance plays a key role in controlling the water-carbon balance of plants) than in more mesic environments (e.g. botanical gardens).

Recently, caution was raised about the use of dual isotope models in some scenarios (Roden & Farquhar 2012), especially when differences in vapour pressure and relative humidity are involved between test conditions. This was not the case in our study, and the strong correlation found between leaf stable isotope compositions and leaf gas exchange rates validate the use of the dual isotope model for our objectives. Nevertheless, caution should be taken when performing studies at global scales or comparing species from different regions.

Isotopes and water sources

In May 2007, soil water $\delta^{18}O$ showed a steep vertical gradient (Fig. 4, inset) indicating that the remaining water in upper soil layers was subjected to strong evaporative isotopic fractionation during this dry and warm period. Stem water $\delta^{18}O$ differed markedly among species at this time (Fig. 4), which suggests partitioning of soil water resources among coexisting plant species. On the contrary, the mesic and cool conditions prevailing in April 2010 were not conducive to the development of a vertical gradient in soil water $\delta^{18}O$ with depth, which explains the largely overlapping stem water $\delta^{18}O$ values among coexisting species at this time.

We found that stem water $\delta^{18}O$ in May 2007 was positively related to stomatal conductance, and negatively related to WUE_i, leaf cellulose $\Delta^{18}O$ and $\delta^{13}C$ across species. This indicates an important relationship between the depth of water extraction and leaf physiological traits in this Mediterranean ecosystem. Plant species exhibiting a more "conservative" water use strategy (low g_s, high WUE_i during peak growing season) are capable of extracting deeper soil water sources (> 40cm depth) during a dry period (e.g. *O. europaea, P. lentiscus, C. humilis, P. halepensis*). In

contrast, plant species showing a more "profligate/opportunistic" water use pattern (high g_s, low WUE_i during peak growing season) maintain a heavy reliance on shallow soil water (<40cm depth) even during dry periods (e.g. *H. syriacum, A. cytisoides, R. officinalis*).

Negative correlations between stem water $\delta^{18}O$ and leaf $\delta^{18}O$ confirmed that inter-specific variation in oxygen isotope composition of leaves primarily reflected differences in stomatal conductance among coexisting species, rather than differences in source water $\delta^{18}O$. Whereas differences in source water $\delta^{18}O$ could also lead to differences in leaf oxygen isotopic composition among co-occurring plant species, this would translate into positive (rather than negative) correlations between stem water $\delta^{18}O$ and leaf $\delta^{18}O$ (see Barbour 2007).

Across species, stem water $\delta^{18}O$ was negatively associated with mean plant size in May 2007, thus indicating that larger species were better capable of extracting isotopically depleted water from deeper soil layers during a dry period. In addition, larger plant species tended to show lower g_s and higher WUE_i during peak growing season than smaller species in this Mediterranean ecosystem (Medrano, Flexas & Galmés 2009). Multiple, inescapable tradeoffs appear to exist between plant size, depth of water uptake, g_s and WUE_i, which might be related to increasing hydraulic constraints with increasing species height (e.g. Ambrose, Sillett & Dawson 2009, Brooks *et al.* 1997, Ryan, Phillips & Bond 2006, Saura-Mas & Lloret 2010).

A "profligate/opportunistic" water use strategy characterized by high g_s and low WUE_i appears adaptive for small-sized plant species (e.g. drought-deciduous or semi-deciduous shrubs, tussock grasses), which are heavily dependent on a highly fluctuating, shallow soil water pool that is subjected to rapid evaporation and uptake by competitors in semiarid habitats (Peñuelas *et al.* 2011, Schwinning & Ehleringer 2001). Profligate water use patterns allow these species to maximize photosynthesis and growth during the relatively narrow windows of opportunity when moisture availability in upper soil layers is optimal following rain pulses (Hernández, Pausas & Vilagrosa 2011, Orians & Solbrig 1977). However, the trade-off of this "profligate/opportunistic" strategy is an early onset of drought dormancy and/or drought deciduousness to escape stress associated with water deficits during unfavourable dry periods (drought avoidance), and therefore a shorter growing season. Drought-deciduousness has been associated with shallow root systems in Mediterranean California (Ackerly 2004). In contrast, conservative water use strategies characterized by tight stomatal control of transpiration and high WUE_i are more advantageous for larger sized species (e.g. evergreen sclerophyll shrubs and trees) capable of reaching more temporally stable, deeper soil water pools that are less exposed to evaporation, and for which competition is less severe. A drought-tolerant, conservative water use pattern may allow species to benefit from occasional rainfall pulses during the dry season, when their more shallow-rooted competitors are dormant (Chesson *et al.* 2004, West *et al.* 2012).

It is important to note that there is a continuous nature of the ecophysiological traits we investigated among the co-occurring species in this semiarid Mediterranean ecosystem. Such a finding is in agreement with other reports of "continuous functional tradeoffs" seen for other plant species and other traits (e.g. Angert *et al.* 2009, West *et al.* 2012, Wright *et al.* 2004). Some plant species (e.g. *R. lycioides*) exhibited a combination of traits that in turn placed them at intermediate positions along our proposed ecophysiological gradient between "profligate/opportunistic" and "conservative" species strategies.

The diversity of water use strategies in this semiarid ecosystem appears to be related to plant species evolutionary history. Many of the woody plant species with a "profligate/opportunistic" water use strategy (*H. syriacum*, *A. cytisoides*, *R. officinalis*) are Quaternary taxa belonging to lineages that evolved in the region after the establishment of Mediterranean climatic conditions (Table 1). In contrast, many of the woody plant species showing conservative water use strategies (*P. lentiscus, O. europaea, C.*

humilis, N. oleander) are pre-Mediterranean, evergreen sclerophyll shrubs belonging to lineages that evolved in tropical-like environments during the Tertiary, and which appeared in the region before the establishment of Mediterranean-type climatic conditions (Peñuelas, Lloret & Montoya 2001, Valiente-Banuet *et al.* 2006). Filella & Peñuelas (2003) highlighted the importance of evolutionary history (and associated root and leaf functional traits) in shaping the strategies of Mediterranean woody plants to cope with summer drought. In this semiarid ecosystem, the plant cover in the more xeric parts of the landscape (ridges, south-facing slopes) is overwhelmingly dominated by Quaternary woody species (as well as by the tussock grass *S. tenacissima*). By contrast, the pre-Mediterranean, Tertiary species are less abundant in this ecosystem, and are largely confined to more mesic parts of the landscape (valley bottoms, dry ravines, north-facing slopes). This suggests that Quaternary species with a "profligate/opportunistic" water use strategy may be better adapted to endure the severe water stress typical of drier habitats (Saura-Mas & Lloret 2010), and may thus be able to perform better under projected climate change scenarios of decreased rainfall and increased temperature for the Mediterranean region.

In conclusion, our results indicate that stomatal regulation intensity and water use strategy are inherent traits of plant species that are conserved across years with contrasting climatic conditions, and can thus be considered major components of functional diversity in semiarid plant communities. Coexisting plant species in this Mediterranean ecosystem segregate along a wide ecophysiological spectrum of contrasting water use strategies, which is reflected in their leaf C and O isotope ratios. According to ecological theory, contrasting (and potentially complementary) water use patterns should foster plant species coexistence and ecosystem primary productivity in Mediterranean habitats, through temporal and spatial partitioning of the resource that is most limiting to vegetation (i.e. soil moisture), as well as through increased total resource capture (Hooper 1998, Hooper *et al.* 2005, O'Connor, Haines & Snyman 2001). Further, a high diversity of water use strategies among coexisting plant species should allow niche segregation of species along subtle, fine-scale ecohydrological gradients as recently discussed by Araya *et al.* (2011) and West *et al.* (2012). This study shows that systematic screening of plant species C and O isotope ratios can be useful to characterize the diversity of water use strategies present in severely water-limited ecosystems. A better understanding of the water use patterns of coexisting species will help researchers make better informed predictions about future shifts in the composition and structure of dryland plant communities in response to forecasted climate changes.

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CAPÍTULO 3

STAND STRUCTURE MODULATES THE LONG-TERM VULNERABILITY OF *PINUS HALEPENSIS* TO CLIMATIC DROUGHT IN A SEMIARID MEDITERRANEAN ECOSYSTEM

Stand structure modulates the long-term vulnerability of *Pinus halepensis* to climatic drought in a semiarid Mediterranean ecosystem

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ABSTRACT

We investigated whether stand structure modulates the long-term physiological performance and growth of *Pinus halepensis* Mill. in a semiarid Mediterranean ecosystem. Tree radial growth and carbon and oxygen stable isotope composition of latewood ($\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$, respectively) from 1967 to 2007 were measured in *P. halepensis* trees from two sharply contrasting stand types: open woodlands with widely scattered trees vs. dense afforested stands.

In both stand types, tree radial growth, $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ were strongly correlated with annual rainfall, thus indicating that tree performance in this semiarid environment is largely determined by interannual changes in water availability.

However, trees in dense afforested stands showed consistently higher $\delta^{18}O_{LW}$ and similar $\delta^{13}C_{LW}$ values compared to those in neighbouring open woodlands, indicating lower stomatal conductance and photosynthesis rates in the former, but little difference in water use efficiency between stand types. Trees in dense afforested stands were more water stressed and showed lower radial growth, overall suggesting greater vulnerability to drought and climate aridification compared to trees in open woodlands.

In this semiarid ecosystem, the negative impacts of intense inter-tree competition for water on *P. halepensis* performance clearly outweigh potential benefits derived from enhanced infiltration and reduced runoff losses in dense afforested stands.

Key words: Competition, $\delta^{18}O$, $\delta^{13}C$, stomatal conductance, water use efficiency, tree-ring, climate change, plant–climate interactions.

INTRODUCTION

Climate change scenarios predict large increases in temperature and decreases in precipitation by the end of the 21st century in the Mediterranean region (Giorgi & Lionello 2008). Greater drought and heat stress associated with climate change have been already related to unprecedented episodes of forest decline (Peñuelas, Lloret & Montoya 2001; Martínez-Vilalta & Piñol 2002;

Linares, Camarero & Carreira 2009; Allen et al. 2010). In the Mediterranean region, extensive reforestation with pines (3.5 million ha reforested with conifers since 1940 in Spain) together with land abandonment have lead to the establishment of dense, uniform, early successional forest vegetation over large areas that may be particularly vulnerable to climate change (Cortina et al. 2011). Understanding how plant community structure modulates the physiological response of trees to drought can help forest managers to adopt strategies for improving the resistance and resilience of these forests to the predicted increase in climatic stress. This is especially important for *Pinus halepensis* Mill. plantations in semiarid areas of the Mediterranean region, where this species has been extensively used in afforestation programmes due to its remarkable ability to withstand drought stress (Maestre & Cortina 2004; Cortina et al. 2011).

In dense afforested plantations, intense inter-tree competition for soil moisture and significant canopy interception of precipitation (Chirino et al. 2006) may exacerbate the severity of water stress experienced by trees. Linares, Camarero & Carreira (2010) found that Mediterranean fir (*Abies pinsapo*) trees already suffering from intense inter-tree competition (a long term stress) were predisposed to decline during severe drought episodes. Thinning experiments have highlighted the importance of inter-tree competition for soil water in semiarid environments (McDowell et al. 2006; Moreno-Gutiérrez et al. 2011). Stand density reductions with thinning have been extensively used to enhance tree water status and growth by means of increasing soil water availability, which is achieved as a consequence of reduced stand transpiration and canopy interception of precipitation (Aussenac & Granier 1988; Brèda, Granier & Aussenac 1995; Misson, Vincke & Devillez 2003). However, improved water status is not always evident in the remaining trees, and some studies have reported no effect of thinning on leaf water potential (Cregg, Hennessey & Dougherty 1990; Schmid et al. 1991). Moreover, several studies conducted in water-limited conifer forests found no evidence for greater drought-induced tree mortality in high density stands under prolonged drought conditions (van Mantgem & Stephenson 2007; Clifford et al. 2008; van Mantgem et al. 2009; Floyd et al. 2009; Ganey & Vojta 2011). These findings question the widely held assumption that increased stand densities in semiarid conifer woodlands predispose trees to experience more intense drought stress during periods of water shortage.

In fact, dense afforested stands are characterized by enhanced runoff infiltration and retention capacities, decreased runoff losses and more mesic microclimate compared to adjacent areas (Van Dijk & Keenan 2007, Aussenac 2000). Recently, Gea-Izquierdo et al. (2009) reported that radial growth in *Quercus ilex* L. was becoming more sensitive to summer drought in the last decades, especially in low density stands, and suggested that denser stands could buffer the influence of extreme climatic events on tree performance. In semiarid ecosystems, the benefits of canopy closure might buffer or offset the negative effects of intense inter-tree competition for water on tree performance in dense afforested stands, depending on annual climatic conditions.

Dendroecological methodologies allow the long-term study of plants performance and their interaction with changing climate. The stable isotope composition of wood provides insight into the ecophysiological processes involved in the response of trees to past environmental conditions. Plant carbon stable isotope composition ($\delta^{13}C$) provides a time-integrated proxy of plant intrinsic water use efficiency (WUE_i) during the growing season (Farquhar, Ehleringer & Hubick 1989; Dawson et al. 2002; Klein et al. 2005). Plant intrinsic water use efficiency is determined by the ratio between photosynthetic rate (A) and stomatal conductance (g_s). In dry environments this ratio is dominated by water availability (which will determine changes in g_s and subsequently in A), and tree ring $\delta^{13}C$ has been found to be strongly and negatively correlated with atmospheric relative humidity and precipitation amount

(Saurer, Siegenthaler & Schweingruber 1995; McCarroll & Loader 2004).

The oxygen stable isotope composition ($\delta^{18}O$) of tree rings is influenced by the $\delta^{18}O$ of source water, the evaporative enrichment at the leaf level, the biochemical fractionation during sucrose formation in the leaves and the re-exchange of oxygen atoms with xylem water during heterotrophic cellulose synthesis (Roden, Lin & Ehleringer 2000; McCarroll & Loader 2004). The evaporative enrichment of leaf water is strongly affected by changes in g_s (Barbour 2007; Farquhar, Cernusak & Barnes 2007) and produces a strong signal that is reflected in the $\delta^{18}O$ values of tree rings. Many studies have found that $\delta^{18}O$ values in tree rings are related to water availability during the growing season (Saurer et al. 1997) and are significantly correlated with precipitation amount, relative humidity and vapour pressure deficit (McCarroll & Loader 2004; Ferrio & Voltas 2005; Reynolds-Henne, Saurer & Siegwolf 2009; Hilasvuori & Berninger 2010). Furthermore, the combined analysis of plant $\delta^{18}O$ and $\delta^{13}C$ can provide valuable complementary information, as $\delta^{18}O$ is related to g_s but is unaffected by A, and can thus help separate the independent effects of A and g_s on $\delta^{13}C$ (Scheidegger et al. 2000; Dawson et al. 2002; Barbour 2007; Battipaglia et al. 2007; 2009; Grams et al. 2007; Querejeta et al. 2006; 2007; 2008).

The aim of this study is to evaluate whether stand structure modulates the long-term physiological performance and growth of *Pinus halepensis* trees in a semiarid Mediterranean ecosystem. We measured tree-ring widths (TRW) and the oxygen and carbon isotopic composition of latewood ($\delta^{18}O_{LW}$ and $\delta^{13}C_{LW}$, respectively) from 1967 to 2007 in two types of stands with sharply contrasting structure and density: a dense afforested plantation, and neighbouring open woodlands with widely scattered pine trees and a well-developed shrub understory. We hypothesized that inter-annual rainfall variability would be the major control on tree physiological status and radial growth in both stand types in this semiarid ecosystem. Second, we hypothesized that pines in dense afforested stands would be consistently more water stressed than those in neighbouring open woodlands due to more intense inter-tree competition for soil water in the former, regardless of large interannual climate variability during the 40 year period evaluated. Since *P. halepensis* is a drought-avoiding, isohydric species with tight stomatal control of transpiration and photosynthesis (Ferrio et al. 2003; Borghetti et al. 1998), we predicted that trees in afforested stands would show chronically lower g_s, A and radial growth and higher $\delta^{18}O_{LW}$ than those in open woodlands, with little difference in $\delta^{13}C_{LW}$ between stand types.

MATERIALS AND METHODS

Study sites

The study was conducted near the city of Murcia (SE Spain) in a dense 60 year old *Pinus halepensis* Mill. plantation and in a nearby open woodland with scattered *P. halpensis* trees. The open woodland has an understory dominated by *Stipa tenacissima* L., *Rosmarinus officinalis* L. and *Anthyllis cytisoides* L. The plantation had an initial density of approximately 1150 trees ha^{-1} but was thinned in 2004 to a final density of 770 trees ha^{-1}. In sharp contrast to afforested stands, tree density in neighboring open woodland stands is less than 20 trees ha^{-1}. The terrain in the experimental area is hilly, with low hills (140-170m asl., < 20 % slopes) and dry ravines between them. The climate is semiarid Mediterranean, with mean annual precipitation of 288 mm and an average annual temperature of 19ºC (Fig. 1; data from the Spanish National Meteorological Agency, AEMET). Soils in the area are mostly haplic calcisols, with some lithic leptosols (according to FAO classification).

Sampling

In May 2008, 10 trees were sampled in each stand type (20 trees in total; mean tree ages are shown in Table 1). EPS values (*Expressed Population Signal*, see following section) were used to assess whether the

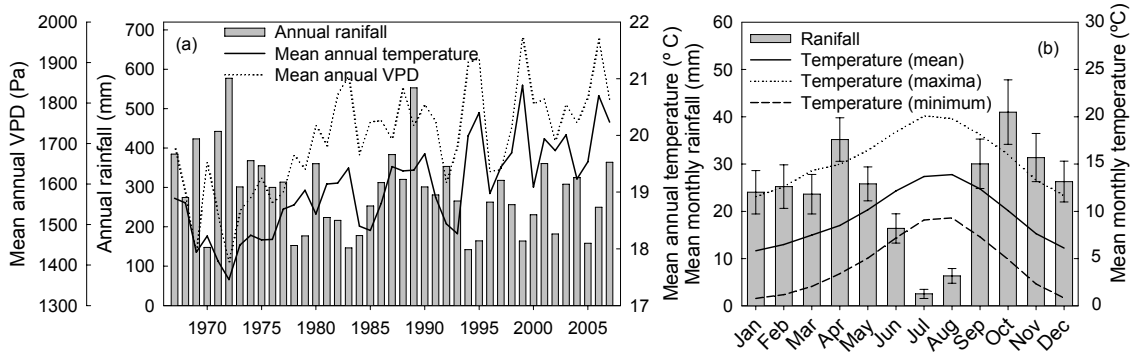

Figure 1. (a) Annual rainfall, mean annual temperature and mean annual vapour pressure deficit (VPD) from 1967 to 2007; (b) Mean monthly rainfall and mean monthly temperatures (maxima, mean and minimum temperatures) for the period from 1967 to 2007, error bars represent ± 1SE. Data from "Embalse de Santomera" weather station in Murcia, provided by the Spanish National Meteorological Agency (AEMET).

samples were representative for building a population chronology. Dominant trees located in the valley floors of independent micro-catchments were chosen for sampling. Three cores per tree were sampled with an increment borer (Haglöf, Långsele, Sweden) of 0.5cm in diameter. The cores were collected at 20cm height, in order to sample the maximum possible number of tree rings, and oriented at 90º to each other, trying to avoid compression wood (Schweingruber 1988). Samples were air dried and sanded for later analyses of tree rings.

In April 2010, leaf gas exchange and stem water potential measurements were performed at midday in 8 and 10 trees of open woodland and the afforested stands, respectively. From the same trees, we also collected lignified twig sections (2 per tree, approximately 10 mm in diameter and 20 mm long) for stem water extractions. After collection, samples were immediately placed in capped vials, wrapped with Parafilm, and stored in the freezer until water extraction.

Dendroecological analysis

As with other Mediterranean species (Cherubini et al. 2003), tree-ring dating in *P. halepensis* was difficult but possible. Tree rings from all cores (three cores per tree) were visually cross-dated (Raventós et al. 2004) and measured to the nearest 0.01mm with a measuring table (LINTAB, Frank Rinn, Heidelberg, Germany) coupled with the TSAP software package (Frank Rinn, Heidelberg, Germany; Rinn, 1996). Cross-dating was statistically verified using the programs TSAP (by the Gleichläufigkeit, GLK: percentage of slopes intervals with equal sign in two time series) and COFECHA (Holmes 1983). Single-core ring-width series were cross-dated with the mean of all individual tree growth series from the same stand type. GLK values were always significant ($P < 0.01$) and higher than 60%. In each stand type, individual tree growth series were standardized with ARSTAN (Cook & Holmes 1984; Holmes 2001) using a two-step detrending after stabilizing the variance ("Briffa/Osborn" variance adjusted version, computed in ARSTAN; Osborn, Briffa & Jones 1997): first, a negative exponential function was applied and secondly, a cubic smoothing spline with a 50% frequency response over 25 years. Afterwards, an autoregressive model was applied to remove the autocorrelation with the previous year ring width. Individual series within each stand type were averaged with a robust (bi-weight) estimation of the mean (Cook 1985). The following parameters were calculated within each stand type: EPS (*Expressed Population Signal*; indicates the level of coherence of the constructed chronology and how it portrays the hypothetical perfect population chronology), r-bar (mean correlation among all possible pairings of individual series within a chronology) and MS (*mean sensitivity*, indicates the degree to which

Characteristics	Open woodland	Afforested plantation
Mean tree age and SE in 2007	76.6 (8.5)	59.5 (0.4)
Mean DBH and SE in 2007	38.1 (3.3)	26.3 (1.2)
Mean EPS (residual)	0.890	0.980
Mean Rbar (residual)	0.427	0.675
Mean sensitivity (residual)	0.375	0.510
Standard deviation (residual)	0.355	0.497
1st order partial autocorrelation (residual)	0.142	0.126

Table 1. Mean tree age (years), mean diameter at breast height (DBH; cm) and dendrochronological characteristics of the residual mean chronology (calculated with ARSTAN, Holmes 2001) of *Pinus halepensis* in open woodland and afforested stands.

TRW changes from year to year and how it is influenced by high-frequency climatic variations). Standardized residual values (TRW_{res}) were used for assessing correlations with climatic data. In order to perform tree radial growth comparisions between stand types, we calculated basal area increments (BAI), which remove variations in radial growth attributable to size and age effects (Van Deusen 1992, Piovesan et al. 2008, Linares et al. 2009). BAI values of individual trees from 1967 to 2007 were calculated using the formula: $BAI = \pi (r^2_t - r^2_{t-1})$, where r is the tree radius and t is the year of tree-ring formation.

Tree rings cellulose extraction and isotopic analysis

The stable isotope composition of tree rings formed between 1967 and 2007 was measured with annual resolution on cellulose extracted from latewood of individual trees (5 trees per stand type). Latewood was carefully split from earlywood under a stereomicroscope. Earlywood was not analyzed for stable isotopes due to possible influence of compounds formed during the previous year (Hill et al. 1995; Robertson et al. 1996). Latewood of the same year from two cores per tree was pooled together. There was not enough wood to conduct isotopic analyses for years 1994, 1995 and 2003.

The cellulose was extracted with a double step digestion, with a 5% NaOH solution at 60ºC for 2 hours followed by a 7% $NaClO_2$ + acetic acid solution at 60ºC for a minimum of 36 hours (Rinne et al. 2005; Boettger et al. 2007; Battipaglia et al. 2008).

Isotopic analyses were conducted at the Stable Isotope Facility at Paul Scherrer Institut (Switzerland). $\delta^{18}O$ was measured using a continuous-flow pyrolysis system (Saurer et al. 1998). $\delta^{13}C$ was determined using an elemental analyzer linked to an isotopic ratio mass spectrometer (MS, Delta S, Finnigan Mat, Germany) via a Conflo II interface (Finnigan Mat, Germany). Isotopic compositions are expressed in delta notation (‰) relative to an accepted reference standard: VPDB for carbon isotope values and VSMOW for oxygen isotope values. The standard deviation for the repeated analysis of an internal standard (commercial cellulose) was better than 0.1‰ for carbon and better than 0.3‰ for oxygen.

Data provided by Francey et al. (1999) and McCarroll & Loader (2004) were used to remove the decline in the $\delta^{13}C$ of atmospheric CO_2 due to fossil fuel emissions from the carbon isotope data series. The corrected series were then employed in all the statistical analyses.

Stem water content and isotopic composition

Stem water from lignified twigs collected in April 2010 was extracted using a cryogenic vacuum distillation line (Ehleringer, Roden & Dawson 2000), and stem water content was calculated gravimetrically. In semiarid ecosystems, inter-plant differences in stem water content often reflect differences in plant water status (e.g. plant water potential; Querejeta, Egerton-Warburton & Allen 2009). Analysis of stem water $\delta^{18}O$ ($\delta^{18}O_{stem\ water}$) was conducted at the Center for Stable Isotope Biogeochemistry, University of

California-Berkeley (USA) by equilibrium of a 0.2ml sample of stem water with an atmosphere of 0.2% of CO_2 for 48h at room temperature (21-23ºC), using a continuous flow isotope ratio mass spectrometer (Finnigan MAT Delta Plus XL; Thermo Instruments Inc., Bremen, Germany). The long-term external precision was ± 0.12‰. $\delta^{18}O$ values are expressed in delta notation (‰) relative to the international standard VSMOW.

Gas exchange and water potential measurements

Net photosynthetic rate (A) and stomatal conductance (g_s) were measured in April 2010 with a portable photosynthesis system (LI-6400, LICOR, Inc., Lincoln, NE, USA) equipped with a LI-6400-40 Leaf Chamber Fluorometer and a LICOR 6400-01 CO_2 injector. *P. halepensis* trees show maximum physiological activity during spring (Maseyk et al. 2008), so leaf gas exchange measurements were taken in April when differences between stand types are expected to be greatest. Gas exchange was measured on 1-year-old, fully sun-exposed needles from intact, attached (non-excised) shoots from the low-middle part of the tree crown (approx. 2m height). Approximately twenty attached needles were placed in a 2 cm^2 leaf cuvette for gas exchange measurements. The CO_2 concentration in the cuvette was maintained at 380 µmol mol^{-1} CO_2. Measurements were done at saturating light of 1.500 µmol m^{-2} s^{-1}, and at ambient air temperature and relative humidity. The leaf-to-air water vapour pressure difference ranged between 0.75 and 1.2 mmol mol^{-1} for all measurements, and the air flow was set to 350 µmol s^{-1}. All leaf gas exchange measurements were conducted at mid-morning between 9:00-11:00 h (local standard time; 7:00-9:00 GMT) on sunny days. Pine needles were collected after leaf gas exchange measurements, and the leaf sections enclosed in the leaf cuvette of the LI-COR 6400 were digitized by scanning on A3 flatbed scanner (HP Deskscan) fitted with a transparency adaptor at 300 dpi, using an 8-bit grayscale. We analyzed the images with specific software (WinRhizo, Regent Instruments Inc., Québec, Canada) to obtain needle surface area (and needle average diameter; Li, Kräuchi & Dobbertin 2006; Fuentes et al. 2007). Total needle surface area values measured by this method were on average 7.5% higher (2.15 cm^2) than the area of the leaf cuvette (2.00 cm^2). All gas exchange parameters were expressed on a total needle surface area basis. Intrinsic water-use efficiency (WUE_i) was calculated as A/g_s.

Stem water potential was measured at midday with a pressure chamber device (Scholander et al. 1965) in 3 small twigs per tree. The twigs had been previously covered with aluminium foil and enclosed in plastic bags to prevent transpiration.

Enhanced Vegetation Index at the stand level

We obtained Enhanced Vegetation Index (EVI) values for each stand type from the Moderate Resolution Imaging Spectroradiometer (MODIS) satellite, and used them as a surrogate of stand transpiration. EVI was extracted from the MOD13Q1 land product which represents 16-days composites of EVI values for a pixel size of 250m. Data for each stand type from October 2000 to September 2007 were downloaded using the 'MODIS Global Subsets: Data Subsetting and Visualization' tool at the ORNL-DAAC (http://daac.ornl.gov/). In drylands, EVI has been shown to be well correlated with structural vegetation properties (e.g. leaf area index, fractional projective cover) and physiological processes directly related to PAR absorption by vegetation (e.g. photosynthesis and transpiration; Glenn et al. 2008; Contreras et al. 2011).

Meteorological data

Meteorological data were provided by the Spanish National Meteorological Agency (AEMET). Monthly values of mean (T), maximum (T_{max}) and minimum (T_{min}) temperatures and precipitation from 1967 to 2007 were obtained from the "Embalse de

Santomera" meteorological station (38º05' N, 1º05' W, 90 masl), located near the sampling sites (<5 km). Missing data were obtained by simple linear regression with the nearby meteorological stations of "Santomera" (38º03' N, 1º02' W, 36 masl) and "Murcia-Alfonso X" (37º59' N, 1º07' W, 90 masl). Atmospheric vapor pressure deficit (VPD) was calculated using the model of Ferrio & Voltas (2005) for the Mediterranean region. From monthly meteorological data we calculated seasonal values (three months periods: January to March, April to June, July to September, October to December), annual values (January to December) and values for the hydrological year (from October of the previous year to September of the current year, oct-Sept) of every measured variable (T, T_{min}, T_{max}, P, VPD).

Statistical analyses

All statistical analyses were performed with SPSS software (version 17.0, SPSS Inc., Chicago). The responses of BAI, $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ from 1967 to 2007 were analyzed with a linear mixed-effect model, with stand type as the main effect (fixed factor), years as the variable to identify repeated observations and an ARMA (first-order autoregressive moving average) covariance structure. The significance of the fixed effect term was assessed with Wald test and likelihood-ratio test. BAI values were log-transformed in order to satisfy assumptions of normality. Individual trees were considered subjects (20 trees for BAI and 10 trees for $\delta^{18}O_{LW}$ and $\delta^{13}C_{LW}$). The effect of stand structure on leaf gas exchange parameters and stem water $\delta^{18}O$ (measured in a single year) were tested using Student's *t*-test. A Wilcoxon matched-pairs signed-ranks test was used to compare EVI values between stand types. Simple Pearson correlations were used to examine the relationships between pairs of measured variables (meteorological variables and TRW_{res}, $\delta^{18}O_{LW}$ and $\delta^{13}C_{LW}$ chronologies) from 1967 to 2007 for each stand type separately. The relationship between A and g_s was assessed across trees from both stand types with simple linear regression.

RESULTS

Tree-ring growth

There was no significant relationship between tree age and mean BAI from 1967 to 2007, either within or across stand types. Mean basal area increment (BAI) from 1967 to 2007 was nearly twice larger in the scattered trees from open woodland stands than in trees from dense afforested stands (11.21 ± 0.50 cm^2/yr vs. 6.68 ± 0.32 cm^2/yr, respectively; P = 0.007; Fig. 2c). Radial growth differences between open woodland and afforested stands seem to have decreased over the last few years, but our chronologies are too short to clearly identify long-term trends in this respect. It should be noted that the afforested stands were thinned in the fall of 2004, which thereafter led to enhanced radial growth in the remaining trees due to competition release (see Moreno-Gutiérrez et al. 2011). In addition, 1994 and 1995 were extremely dry years in which most sampled trees did not show any detectable radial growth (only one tree out of ten in the afforested stands and four trees out of ten in the open woodland stands showed any detectable growth). This exceptionally severe drought may have damaged the trees in both stand types, thus leading to smaller differences in growth between stand types during subsequent years.

The detrended tree ring width chronologies (TRW_{res}) of both stand types were strongly correlated with one another (r = 0.876; P <0.01) during the period from 1967 to 2007 (see Fig. 2d). High EPS values (Table 1; greater than 0.85) in both types of stands indicate that constructed chronologies from detrended individual tree ring width series (TRW_{res}) were representative of radial growth variations of the whole population of trees (Wigley, Briffa & Jones 1984). There was also good coherence among individual growth series (high mean r-bar; Table 1). The r-bar and MS values were higher in the afforested stands than in the open woodland stands (Table 1), thus indicating that in afforested stands there is a stronger common growth signal and greater year-to-year radial growth variability associated to inter-annual changes in climatic conditions. Furthermore,

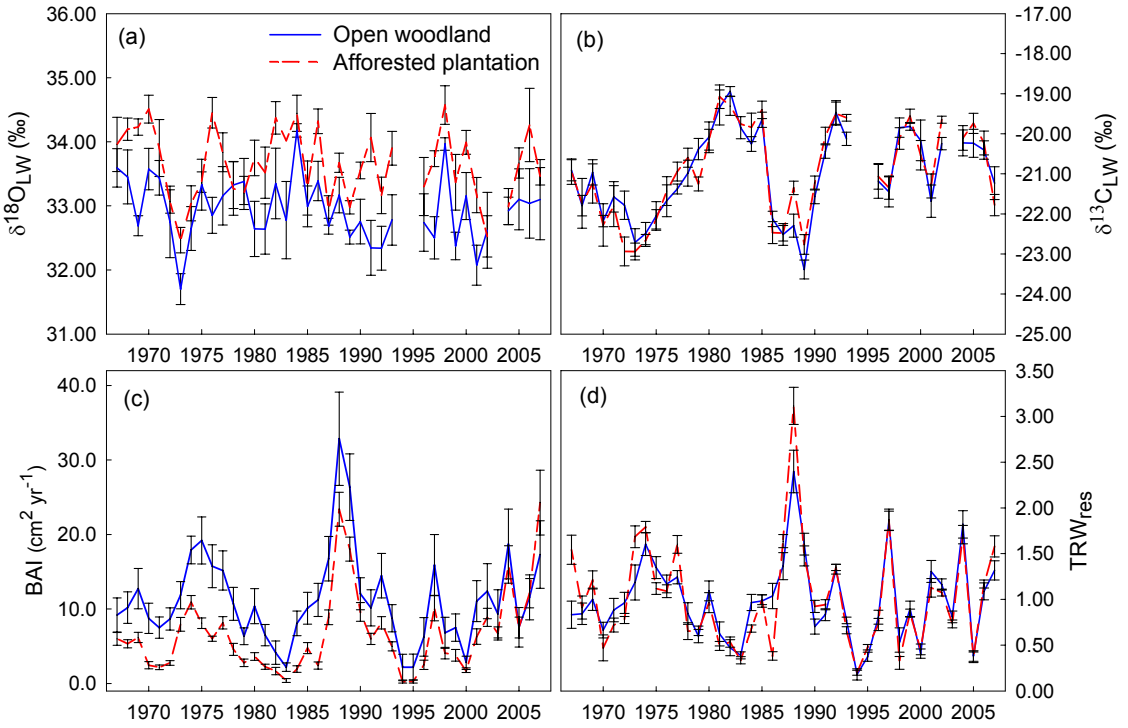

Figure 2. Open woodland and afforested stands mean chronologies of (a) oxygen isotopic composition of latewood ($\delta^{18}O_{LW}$, n=5 in each stand type), (b) carbon isotopic composition of latewood ($\delta^{13}C_{LW}$, n=5 in each stand type), (c) basal area increments (BAI, n=10 in each stand type) and (d) residual tree-ring widths (TRW_{res}, n=10 in each stand type) from 1967 to 2007. The afforested stands were thinned in the fall of 2004 (from 1150 trees ha^{-1} to a final density of 770 trees ha^{-1}; tree density in open woodland stands is less than 20 trees ha^{-1}). $\delta^{13}C_{LW}$ values were corrected according to Francey et al. (1999) and McCarroll & Loader (2004). Error bars represent ± 1SE.

the standard deviation of the residual chronology was larger in afforested than in open woodland stands (Table 1), suggesting that radial growth responses to extreme climatic events are stronger in the former.

Tree-ring stable isotope composition

Latewood carbon isotope composition chronologies ($\delta^{13}C_{LW}$) were tightly coupled between open woodland and afforested stands (Fig.2b) and were strongly correlated with each other for the period 1967 to 2007 ($r = 0.932$, $P < 0.01$). Compared to $\delta^{13}C_{LW}$, latewood oxygen isotope composition chronologies ($\delta^{18}O_{LW}$) showed a significant but weaker correlation between stand types (Fig. 2a; $r = 0.607$, $P < 0.01$, from 1967 to 2007) due to greater variability among the $\delta^{18}O_{LW}$ time series of individual trees.

From 1967 to 2007, pines in open woodland stands showed consistently lower $\delta^{18}O_{LW}$ values than those in afforested stands (Fig. 2a; mean values were 32.95 ± 0.18‰ vs. 33.63 ± 0.18‰, respectively; $P = 0.022$). By contrast, there was no difference in $\delta^{13}C_{LW}$ between both stand types (Fig. 2b).

Within each stand type, correlations of the $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ chronologies with TRW_{res} chronologies were significant or marginally significant and of negative sign (Table 2). $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ chronologies were not significantly correlated with each other within or across stand types (Table 2).

Relationships with climatic variables

Tree-ring chronologies (TRW_{res}, $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$) from both stand types showed similar correlation patterns with meteorological variables for the 1967-2007

Table 2. Pearson's correlation coefficients (r) and p-values (in parentheses) of the relationship between the chronologies of residual tree-ring widths (TRW_{res}) and carbon isotopic composition of latewood ($\delta^{13}C_{LW}$), TRW_{res} and oxygen isotopic composition of latewood ($\delta^{18}O_{LW}$) and $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ from 1967 to 2007. $\delta^{13}C_{LW}$ values were corrected according to Francey *et al*. (1999) and McCarroll & Loader (2004). Chronologies from each stand type as well as the averaged chronology across stand types are considered. Significant values ($P < 0.05$) are highlighted in bold.

	TRW_{res} vs. $\delta^{13}C_{LW}$	TRW_{res} vs. $\delta^{18}O_{LW}$	$\delta^{13}C_{LW}$ vs. $\delta^{18}O_{LW}$
Open woodland	**-0.503 (0.001)**	-0.295 (0.065)	0.135 (0.405)
Afforested plantation	-0.288 (0.072)	**-0.406 (0.009)**	0.198 (0.220)
Both stand types	**-0.398 (0.011)**	**-0.411 (0.008)**	0.177 (0.276)

period. TRW_{res} was strongly positively correlated with precipitation in both stand types (Figs. 3a and 4a). TRW_{res} was particularly strongly correlated with precipitation of the whole hydrological year (from October of the previous year until September of the current year) in both stand types. TRW_{res} was also strongly affected by water availability during spring months, as shown by tight correlations with total precipitation of spring (April to June; Figs. 3a and 4a). Relationships of TRW_{res} with temperature and atmospheric vapor pressure deficit (VPD) were not significant, but they were mainly of negative sign (Figs. 3a and 4a). In both stand types, TRW_{res} was marginally negatively correlated with VPD of July (Figs. 3a and 4a), thus indicating that tree-ring growth in *P. halepensis* is also influenced by weather conditions during the summer months. Minimum temperature of January (the coldest month; Fig. 1) was marginally positively correlated with TRW_{res} ($r = 0.300$, $P = 0.057$, $n = 41$ in open woodland stands; $r = 0.299$, $P = 0.058$, $n = 41$ in afforested stands), thus suggesting that mild winter temperatures enhance radial growth in *P. halepensis*.

$\delta^{13}C_{LW}$ was negatively correlated with precipitation, while it was positively correlated with temperature and VPD in both stand types (Figs 3b and 4b). $\delta^{13}C_{LW}$ was strongly correlated with precipitation of the whole current hydrological year (oct-Sept) in both stand types, and also with precipitation during several particular periods within the growing season (best correlations with precipitation of March, September and autumn; Figs 3b and 4b). $\delta^{13}C_{LW}$ was positively correlated with mean VPD during the whole hydrological year, and during several months of spring and autumn (Figs 3b and 4b). In both stand types, $\delta^{13}C_{LW}$ was positively correlated with mean temperature of April (Figs 3b and 4b) and of the whole hydrological year (oct-Sept, although this correlation is only marginally significant in open woodland stands).

Although inter-tree variability in $\delta^{18}O_{LW}$ values was high, the mean $\delta^{18}O_{LW}$ chronologies from both stand types still contained a clear climatic signal, even if correlations with meteorological variables were weaker than for $\delta^{13}C_{LW}$. The $\delta^{18}O_{LW}$ chronologies from both stand types were strongly negatively correlated with precipitation of the whole hydrological year (Figs. 3c and 4c). Negative correlations were also found with precipitation of September and summer (July to September) in both stand types (Figs. 3c and 4c). VPD of July strongly influenced $\delta^{18}O_{LW}$ in both stand types (positive correlation; Figs. 3c and 4c). A positive correlation was also found between $\delta^{18}O_{LW}$ and maximum temperatures of July in afforested ($r = 0.366$, $P = 0.024$, $n = 38$) and open woodland stands ($r = 0.511$, $P = 0.001$, $n = 38$). Paradoxically, $\delta^{18}O_{LW}$ was negatively correlated with VPD ($r= -0.340$, $P=0.037$, $n=38$) and T_{max} ($r= -0.379$, $P=0.019$, $n=38$) from April to June in the open woodland stands, but not in the afforested stands.

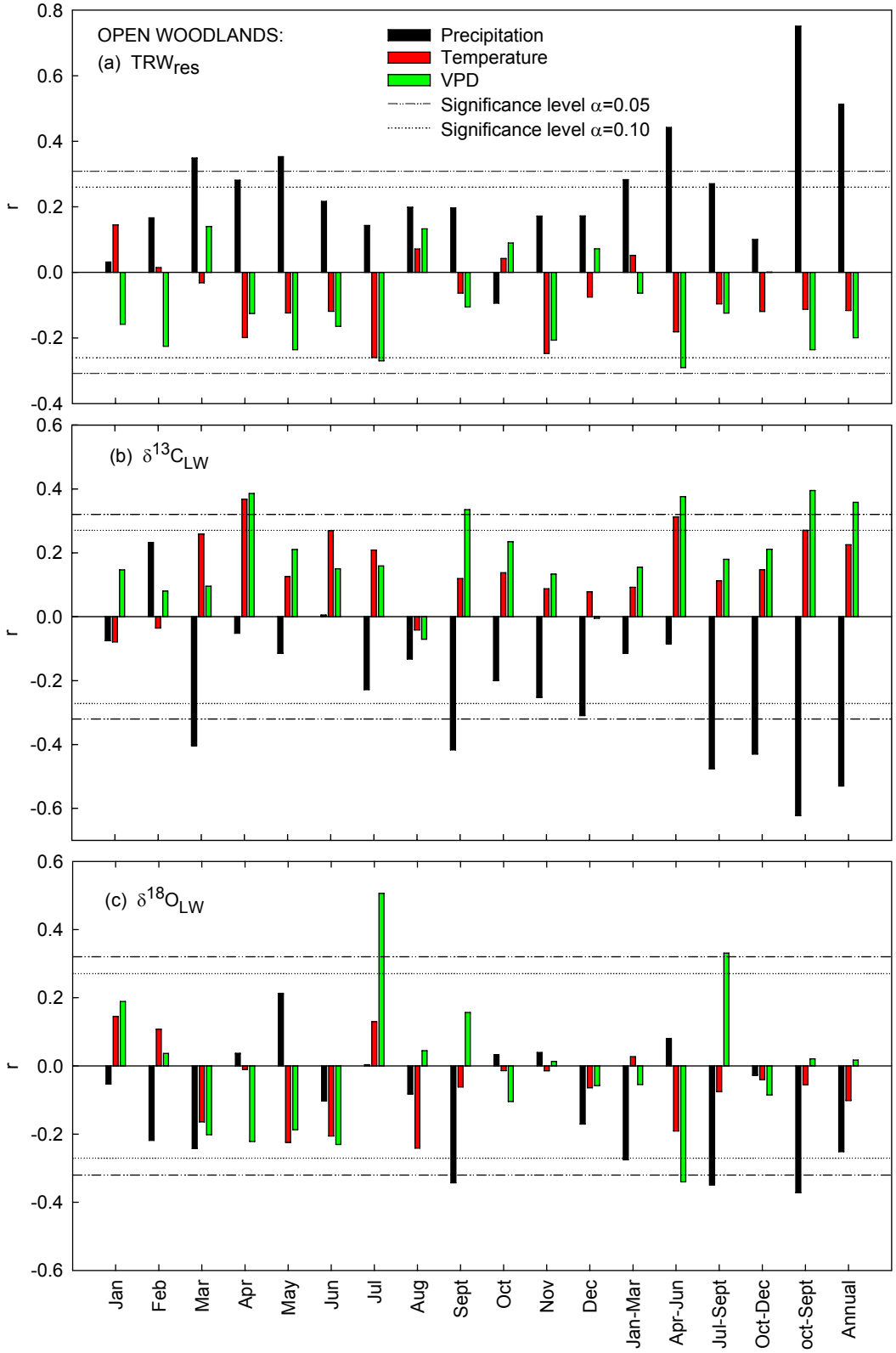

Figure 3. Pearson's correlation coefficients (r) and significance values (dotted lines) for the relationship between monthly/seasonal climatic variables and chronologies of (a) residual tree ring widths (TRW_{res}), (b) $\delta^{13}C_{LW}$ and (c) $\delta^{18}O_{LW}$ in open woodlands from 1967 to 2007. The $\delta^{13}C_{LW}$ values were corrected according to Francey et al. (1999) and McCarroll & Loader (2004). "oct-Sept" represents the hydrological year, from October of the previous year to September of the current year.

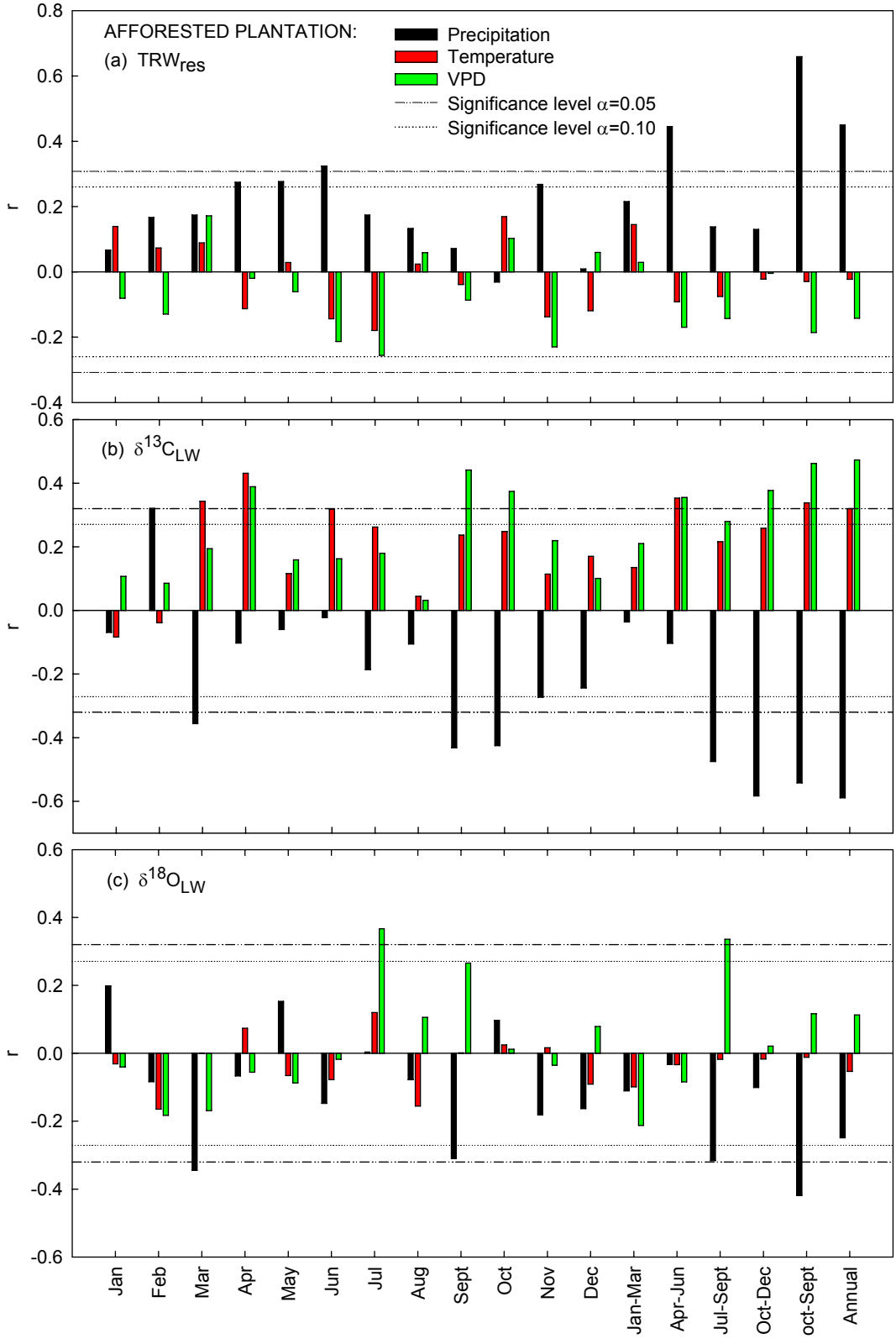

Figure 4. Pearson's correlation coefficients (r) and significance values (dotted lines) for the relationship between monthly/seasonal climatic variables and chronologies of (a) residual tree ring widths (TRW_{res}), (b) $\delta^{13}C_{LW}$ and (c) $\delta^{18}O_{LW}$ in afforested stands from 1967 to 2007. The $\delta^{13}C_{LW}$ values were corrected according to Francey et al. (1999) and McCarroll & Loader (2004). "oct-Sept" represents the hydrological year, from October of the previous year to September of the current year.

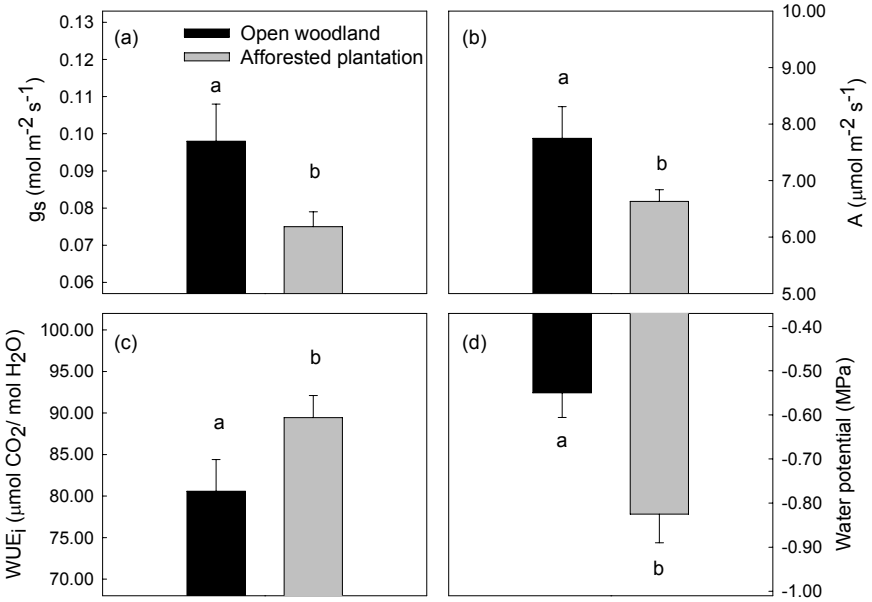

Figure 5. Mean values of (a) stomatal conductance (g_s), (b) net photosynthetic rate (A), (c) intrinsic water use efficiency (WUE_i) and (d) midday stem water potential of *Pinus halepensis* trees from contrasting stand types (n=8 in open woodland stands and n=10 in afforested stands). Differences in g_s and stem water potential between stand types were significant at $P < 0.05$; differences in A and WUE_i between stand types were marginally significant at $P < 0.1$. Error bars represent 1SE.

Stem water potential, content and $\delta^{18}O$ and leaf gas exchange data

Stem water potential at midday was significantly higher (P = 0.007) in trees from open woodlands than in those from afforested stands (Fig. 5d) during the peak growing season (April 2010). Trees in open woodlands also had higher stem water contents than those in afforested stands (51.20 ± 0.82% and 43.84 ±1.04%, respectively; $P < 0.001$). The oxygen stable isotope composition of stem water ($\delta^{18}O_{stem\ water}$) differed between plant communities (P = 0.029), with more enriched values in the afforested stands (-6.46 ± 0.22‰) than in open woodlands (-7.12 ± 0.13‰).

Stomatal conductance (g_s) was significantly higher (P = 0.025) in open woodlands (0.098 ±0.010mol m^{-2} s^{-2}) than in afforested stands (0.075 ± 0.004 mol m^{-2} s^{-2}; Fig. 5a) during peak growing season (April 2010). Photosynthetic activity (A) was also marginally higher in open woodland stands (7.75 ± 0.56 µmol m^{-2} s^{-2}) than in afforested stands (6.63 ± 0.21 µmol m^{-2} s^{-2}; P=0.094, n=18; Fig. 5b). A and g_s were strongly positively correlated with one another (Fig. 6) across individuals from both stand types, thus indicating tight stomatal control of A in *P. halepensis*. Intrinsic water use efficiency (WUE_i) was marginally lower (P = 0.067, n=18; Fig. 5c) in the open woodland stands (80.59 ± 3.80 µmol CO_2/mol H_2O) than in the afforested stands (89.44 ± 2.66 µmol CO_2/mol H_2O).

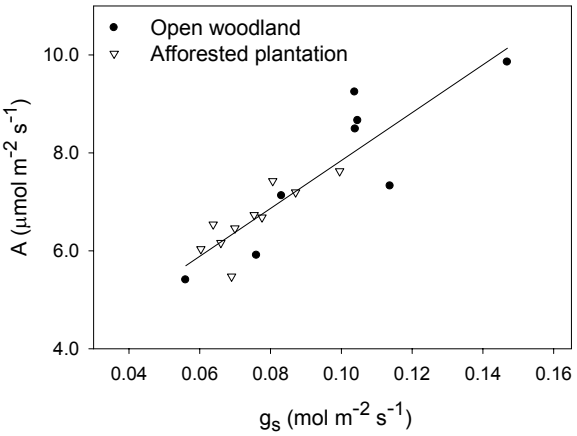

Figure 6. Relationship between net photosynthetic rate (A) and stomatal conductance (g_s) in *Pinus halepensis* trees across stand types ($r = 0.892$, $P < 0.01$, $r^2 = 0.796$; n=18; $A = 2.95 + 48.88 g_s$).

Enhanced Vegetation Index (EVI)

Mean annual values of EVI were consistently higher (≈30% on average, $p<0.001$) in the afforested area (0.234 ± 0.005) than in the open woodland (0.178 ± 0.004), with smallest (15.5%) and largest (40.9%) differences between stand types observed during spring and summer, respectively.

DISCUSSION

Major climatic controls on P. halepensis ecophysiology

In severely water-limited ecosystems, the physiological performance of *P. halepensis* is strongly dependent on water availability (Ferrio *et al*. 2003; Maseyk *et al*. 2011). Annual TRW_{res} values from both stand types were indeed tightly positively correlated with precipitation of the entire hydrological year (which comprises several months from the previous calendar year; Figs. 3a and 4a). The strong influence of precipitation on tree radial growth clearly overwhelms the influence of temperature in this semiarid environment. Furthermore, in both stand types tree-ring $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ were strongly influenced by inter-annual rainfall variability, and both $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ were in turn negatively correlated with TRW_{res} (Table 2). Overall, these relationships indicate that radial growth of *P. halepensis* trees in this semiarid environment shows a strong positive response to increased rainfall due to enhanced stomatal conductance (g_s) and photosynthesis (A) during wet periods.

In both stand types, TRW_{res} was positively correlated with precipitation from April to June (Figs. 3a and 4a), thus suggesting that radial growth in *P. halepensis* is largely determined by earlywood formation during the wet spring months. $\delta^{13}C_{LW}$ and $\delta^{18}O_{LW}$ were strongly correlated with climatic conditions during March and September, as well as with conditions during the entire autumn (September to December; Figs. 3b,c and 4b,c), which is the time when latewood (the wood fraction that was analyzed for stable isotopes) is laid down in *P. halepensis* (De Luis *et al*. 2007). In a nearby location, De Luis *et al*. (2007) found that *P. halepensis* trees had two main growing periods starting in March and September, respectively. Similar seasonal growth patterns have been reported for *P. halepensis* by Klein *et al*. (2005), De Luis *et al*. (2009), Sánchez-Salguero *et al*. (2010), and Camarero, Olano & Parras (2010).

In semiarid environments, evaporative enrichment of leaf water is expected to dampen the $\delta^{18}O$ signal of precipitation in tree rings of *P. halepensis* (Ferrio & Voltas 2005). $\delta^{18}O_{LW}$ was indeed negatively correlated with rainfall amount in both stand types (Figs. 3c and 4c), thus indicating that $\delta^{18}O_{LW}$ values reflect changes in stomatal conductance in response to changes in water availability. Tree ring $\delta^{18}O_{LW}$ was positively correlated with VPD of July, thus showing that factors other than low soil water availability (like high VPD) can also decrease A and g_s in *P. halepensis* during dry periods (Klein *et al*. 2005). In both stand types, VPD and temperature were positively correlated to $\delta^{13}C_{LW}$ as well, suggesting increased WUE_i due to greater plant physiological stress and reduced stomatal conductance during dry and hot periods.

Despite similar correlations patterns with climatic variables in both stand types, some noteworthy differences suggest that pines in afforested stands are more prone to water shortage than pines in open woodlands. First, the year to year variability of tree ring widths was larger in the afforested plantation (higher mean sensitivity; Table 1), thus indicating that these pines may rely more heavily on current year rainfall than those in open woodland stands. Second, tree ring width was more strongly correlated with $\delta^{18}O_{LW}$ in afforested than in open woodland stands (Table 2), which suggests greater growth dependence on stomatal responses to fluctuations in water availability in the former. Third, during the exceptionally severe drought of 1994-1995, fewer trees showed detectable radial growth in afforested stands than in open woodlands (1 out of 10 vs. 4 out of 10, respectively). Fourth, the paradoxical negative correlation between $\delta^{18}O_{LW}$ and VPD from April to June in the

open woodlands (but not in afforested stands) indicate greater increases in stomatal conductance (that would cause lower isotopic enrichment of leaf water through an enhanced "Péclet effect"; Barbour 2007), and/or greater access to deeper, isotopically depleted water sources (Barbour 2007) during periods of high transpirational demand in spring .

Differences in tree physiological status between stand types

Widely scattered trees in open woodland stands showed consistently lower $\delta^{18}O_{LW}$ values than trees in dense afforested stands during the entire period from 1967 to 2007 (Fig. 2a), regardless of large interannual climate variability during this 40 year period. Lower $\delta^{18}O_{LW}$ values suggest higher stomatal conductance (g_s) in the open woodland stands (Barbour 2007), which is well supported by leaf gas exchange data showing higher g_s values in trees of open woodland stands than in those of afforested stands during peak growing season (Fig. 5a). Higher stem water potential and content in open woodlands than in afforested stands further support that *P. halepensis* trees are considerably less water stressed in the former stand type. Differences in stomatal conductance and in stem water potential and content between contrasting stand types were quite large in April 2010 despite the thinning conducted in 2004 in the afforested stands, which led to substantial competition release for the remaining trees (Moreno-Gutiérrez *et al*. 2011).

In sharp contrast to $\delta^{18}O_{LW}$, there was no significant difference in tree-ring $\delta^{13}C_{LW}$ between stand types during the period between 1967 and 2007 (Fig. 2b), thus suggesting that pines in both systems had roughly similar intrinsic water use efficiencies (WUE_i) (Farquhar *et al*. 1989). Based on the dual isotope model developed by Scheidegger *et al*. (2000) and Grams *et al*. (2007), no difference in $\delta^{13}C_{LW}$ combined with lower $\delta^{18}O_{LW}$ values in open woodland than in afforested stands indicates greater g_s and A in the former. Again, this interpretation of isotopic data is well supported by both leaf gas exchange and tree radial growth data (Figs. 5 and 2c, respectively). Furthermore, the strong positive correlation found between A and g_s across trees from both stand types (Fig. 6) is evidence of tight stomatal control of carbon assimilation rate in *P. halepensis*.

Large differences in the degree of inter-tree competition for water between stand types can explain the lower stem water potential and content, lower stomatal conductance and poorer radial growth of trees in dense afforested stands compared to those in neighbouring open woodland stands. The widely scattered distribution of trees in open woodland stands resulted in lower stand level transpiration compared to dense afforested stands as indicated by lower EVI values in the former (Glenn *et al*. 2008; Contreras *et al*. 2011), thus allowing for a more complete recharge of the soil profile that permitted higher leaf-level stomatal conductance in open woodland stands. Similar results were found in a previous study that compared the physiological performance *P. halepensis* between contrasting stand densities after thinning application (Moreno-Gutiérrez *et al.* 2011), which further supports the notion that differences in tree water status between afforested and open woodland stands are related to differences in tree density.

Similar $\delta^{13}C_{LW}$ values between afforested and open woodland stands suggests that *P. halepensis* maintains in the long-term a homeostatic control of the ratio c_i/c_a (the intercellular to atmospheric CO_2 concentration, which determines $\delta^{13}C_{LW}$ and WUE_i) as an acclimation response to chronic water shortage (McDowell *et al*. 2006; Maseyk *et al*. 2011). In the afforested stands, the "setpoint" for c_i/c_a was accomplished by down-regulation of A through tight stomatal control of transpiration (indicated by higher $\delta^{18}O_{LW}$ and lower g_s in pines of the afforested stands; Figs. 2a and 5a). However, the complicating effects of density-dependent disparities in light and nutrient availability between contrasting stand types (Dawson *et al*. 2002) might have also contributed to the lack of difference in $\delta^{13}C_{LW}$ between them. In April 2010, instantaneous leaf gas exchange measurements showed marginal differences in WUE_i between stand types (Fig. 5), thus

revealing the occurrence of transient differences in water use efficiency between stand types that were not detected at the annual time-scale of $\delta^{13}C_{LW}$ values. Several authors have reported that there is a dampening of the carbon and oxygen isotopic signal of soluble carbohydrates during phloem loading and transport from the leaves to the trunk and/or during heterotrophic cellulose synthesis (Klein *et al*. 2005; Gessler *et al*. 2009; Offermann *et al*. 2011), which can lead to discrepancies between instantaneous leaf gas exchange measurements and the stable isotope composition of tree rings.

In addition to lower g_s, consistently less enriched $\delta^{18}O_{LW}$ values in open woodland compared to afforested stands might also be related in part to more depleted source water $\delta^{18}O$ in the former. As no fractionation occurs during water uptake by plants (Dawson *et al*. 2002), lower $\delta^{18}O_{stem\ water}$ indicated less evaporatively enriched source water for trees in open woodland stands than in afforested stands. As upper soil layers dry up faster in open woodland than in dense afforested stands (Raz-Yaseef, Rotenberg & Yakir 2010), pines in open woodlands may be forced to rely more heavily on water stored in deeper soil layers, which tends to have lower $\delta^{18}O$ than water from upper soil because evaporative isotopic fractionation decreases with soil depth (Dawson *et al*. 2002).

In conclusion, tree-ring growth and $\delta^{18}O_{LW}$, but not $\delta^{13}C_{LW}$, are affected by stand structure in this severely water-limited ecosystem. TRW_{res}, $\delta^{18}O_{LW}$ and $\delta^{13}C_{LW}$ are strongly correlated with total annual rainfall in both afforested stands and open woodlands. However, tree-ring growth and $\delta^{18}O_{LW}$ analysis revealed that widely scattered pines in open woodland stands are consistently less water stressed than those in dense afforested stands. Trees in afforested stands experience more severe water shortage due to intense inter-tree competition for soil moisture, and therefore may be more vulnerable to climatic drought than trees in open woodland stands. In this semiarid ecosystem, the benefits of negligible inter-tree competition for water in open woodland stands clearly outweigh potential benefits that canopy closure might provide to trees in dense afforested stands (e.g. enhanced runoff infiltration and retention, reduced evaporation of soil water due to shading, more mesic microclimate, etc.). In view of the projected increases in the frequency and duration of drought in the Mediterranean basin (Giorgi & Lionello 2008), these results have important implications for the management of *P. halepensis* plantations, which currently cover thousands of hectares in the region. Silvicultural thinning aimed at reducing inter-tree competition for water may alleviate drought stress in the remaining trees, and may help mitigate the adverse impacts of climate aridification on dense conifer plantations. Our results indicate that the influence of stand structure on tree vulnerability to climatic drought should be taken into account when designing afforestation and silvicultural management strategies for drylands, in order to foster the long-term sustainability of semiarid conifer woodlands under projected climate change scenarios.

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CAPÍTULO 4

LEAF $\delta^{18}O$ OF REMAINING TREES IS AFFECTED BY THINNING INTENSITY IN A SEMIARID PINE FOREST

Leaf $\delta^{18}O$ of remaining trees is affected by thinning intensity in a semiarid pine forest

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ABSTRACT

Silvicultural thinning usually improves the water status of remaining trees in water-limited forests. We evaluated the usefulness of a dual stable isotope approach ($\delta^{13}C$, $\delta^{18}O$) for comparing the physiological performance of remaining trees between forest stands subjected to two different thinning intensities (moderate vs. heavy) in a 60 year-old *Pinus halepensis* Mill. plantation in semiarid southeastern Spain. We measured bulk leaf $\delta^{13}C$ and $\delta^{18}O$, foliar elemental concentrations, stem water content, stem water $\delta^{18}O$ ($\delta^{18}O_{stem\ water}$), tree ring widths and leaf gas exchange rates to assess the influence of forest stand density on tree performance. Remaining trees in low-density stands (heavily thinned) showed lower leaf $\delta^{18}O$, and higher stomatal conductance, photosynthetic rate and radial growth than those in moderate-density stands (moderately thinned). By contrast, leaf $\delta^{13}C$, intrinsic water use efficiency, foliar elemental concentrations and $\delta^{18}O_{stem\ water}$ were unaffected by stand density. Lower foliar $\delta^{18}O$ in heavily thinned stands reflected higher stomatal conductance of remaining trees due to decreased inter-tree competition for water, whereas higher photosynthetic rate was largely attributable to reduced stomatal limitation to CO_2 uptake. The dual isotope approach provided insight into the early (12 months) effects of stand density manipulation on the physiological performance of remaining trees.

Key words: *Pinus halepensis*, competition, drought stress, dual isotope approach, stomatal conductance, oxygen isotopic composition.

INTRODUCTION

Pinus halepensis Mill. is a drought-adapted species that has been extensively used in afforestation programmes in semiarid areas of the Mediterranean basin during the last 100 years (Maestre & Cortina 2004). In semiarid southeastern Spain, afforestation with *P. halepensis* has been primarily aimed at preventing catastrophic floods, reducing soil erosion rates and combating desertification. In many instances, afforestation of degraded soils with *P. halepensis* has lead to the establishment of high density plantations with slow-growing, even-aged trees which are prone to fire and pest attacks (Maestre & Cortina 2004). Silvicultural thinning is a suitable and widely used forest management alternative in this

context, as it can enhance resource (light, nutrients, water) uptake and growth in remaining trees (Brèda, Granier & Aussenac 1995; Kolb *et al.* 2007). Thinning in drought-prone forests is primarily aimed at increasing water availability for remaining trees through reduced canopy interception of rainfall and decreased inter-tree competition for soil water (Brèda *et al.* 1995; McDowell *et al.* 2006; Martín-Benito *et al.* 2010). In semi-arid conifer forests, enhanced soil water availability is to a large extent responsible for increased growth of remaining trees after thinning (Klein *et al.* 2005; McDowell *et al.* 2006). Several studies have shown that thinning stimulates *P. halepensis* growth by reducing inter-tree competition for growth-limiting soil resources (water and nutrients; Ne'eman, Lahavb & Izhaki 1995; González-Ochoa, López-Serrano & De Las Heras 2004; Moya *et al.* 2008). However, diameter growth responses to thinning can take several years to become noticeable in semiarid *Pinus halepensis* forests due to very slow tree radial growth. It thus becomes difficult to evaluate the effectiveness of stand density manipulation for improving the performance of remaining trees in these low-productivity forests. Stable isotope measurements in bulk leaf material could help overcome this limitation.

Stable isotope techniques are a powerful research tool in plant ecophysiological studies, as isotopic signatures provide time-integrated information on plant resource uptake and plant responses to the changing biotic and abiotic environments (Dawson *et al.* 2002). In C3 plants, carbon stable isotope composition ($\delta^{13}C$) is inversely and linearly correlated with c_i/c_a, the ratio of intercellular to atmospheric CO_2 concentrations in leaves (Farquhar, Ehleringer & Hubick 1989). This ratio reflects the relative magnitudes of net photosynthetic rate (A) and stomatal conductance (g_s), and thus $\delta^{13}C$ is a good indicator of plant intrinsic water use efficiency (WUE_i), which is given by the ratio A/g_s (Farquhar *et al.* 1989; Dawson *et al.* 2002; Klein *et al.* 2005). The oxygen stable isotope composition ($\delta^{18}O$) of plant organic material is strongly influenced by the isotopic signature of source water, which may change with depth of water uptake due to evaporative isotopic enrichment of soil water near the surface (Dawson *et al.* 2002; Barbour 2007). Plant $\delta^{18}O$ is also inversely related to e_a/e_i, the ratio of atmospheric to leaf intercellular water vapour pressure, and thus is strongly affected by changes in g_s (Barbour 2007; Farquhar, Cernusak & Barnes 2007). Since plant $\delta^{18}O$ is related to g_s but is unaffected by A, it can help separate the independent effects of A and g_s on $\delta^{13}C$ (Barbour 2007): simultaneous measurement of $\delta^{18}O$ and $\delta^{13}C$ in plant tissues allows discrimination between biochemical and stomatal limitations to photosynthesis (Scheidegger *et al.* 2000; Dawson *et al.* 2002; Grams *et al.* 2007; Querejeta *et al.* 2006; 2007; 2008).

$\delta^{13}C$ measurements in plant material (leaves and/or tree growth-rings) have been often used to assess the effects of stand density manipulation on the ecophysiological performance of remaining trees, with different results (Warren, McGrath & Adams 2001; McDowell *et al.* 2003; Sala *et al.* 2005). Reduced competition for water and increased soil water availability after thinning may lead to reduced WUE_i due to differential enhancement of g_s over A in remaining trees (Meinzer, Goldstein & Grantz 1993; McDowell *et al.* 2003; Skov, Kolb & Wallin 2004; Ferrio *et al.* 2005). However, forest thinning can also increase foliar nutrient concentrations (López-Serrano *et al.* 2005) and incident light levels in remaining trees, thus enhancing their A and WUE_i (Warren *et al.* 2001). Increased soil water availability, on the one hand, and enhanced nutrient and/or light availability, on the other, can exert effects of opposite sign on the WUE_i of remaining trees after silvicultural thinning. Unsurprisingly, different studies have found widely different responses of WUE_i and $\delta^{13}C$ to forest thinning. McDowell *et al.* (2003) reported depletion of $\delta^{13}C$ values in *Pinus ponderosa* after forest thinning, due to a greater increase in g_s than in A that lead to reduced WUE_i in remaining trees. By contrast, Warren *et al.* (2001) and Powers, Pregitzer & Palik (2008) reported foliar $\delta^{13}C$ enrichment in various

Pinus species after thinning, which they attributed to differential enhancement of A over g_s due to increased foliar nutrient concentrations and greater canopy light interception in remaining trees. Other studies have reported no change in the $\delta^{13}C$ values of remaining pine trees after thinning (Wallin *et al.* 2004; Sala *et al.* 2005; McDowell *et al.* 2006), allegedly due to roughly parallel increases in both g_s and A, without significant change in WUE_i.

Simultaneous measurement of $\delta^{18}O$ and $\delta^{13}C$ in bulk leaf material could help elucidate which physiological processes (A, g_s, WUE_i) are most strongly affected by stand density manipulation in each particular situation. Huang *et al.* (2008) and Brooks & Coulombe (2009) recently used this dual isotope approach to investigate the effects of various forest management practices (weed control, fertilizer addition) on tree physiological status. However, to the best of our knowledge, only Powers *et al.* (2008) have used both foliar $\delta^{18}O$ and $\delta^{13}C$ to evaluate the effects of silvicultural thinning on the performance of remaining trees. Powers *et al.* (2008) found increased $\delta^{13}C$ and no significant change in $\delta^{18}O$ in response to thinning, which they interpreted as evidence of enhanced A (and to a lesser extent, g_s) in remaining pine trees.

Several recent studies have successfully used $\delta^{18}O$ and $\delta^{13}C$ measurements to investigate the intensity of inter- or intra-specific competition for soil resources among plants (Grams *et al.* 2007; Huang *et al.* 2008; Ramírez, Querejeta & Bellot 2009). The main goal of the present study was to evaluate the usefulness of the dual stable isotope approach for early (≈ 1 year) detection of differences in tree physiological status between forest stands subjected to different thinning levels in a semi-arid environment. We hypothesized that heavy thinning in a 60 year-old *Pinus halepensis* plantation would reduce inter-tree competition for water and would improve the water status of remaining trees to a greater extent than moderate thinning. We predicted that remaining trees in low-density stands (subjected to heavy thinning) would show higher g_s and A, and more depleted foliar $\delta^{18}O$, than those in moderate density stands (subjected to moderate thinning). Further, we expected to find no differences in WUE_i or foliar $\delta^{13}C$ between treatments due to tightly coupled changes in g_s and A. We also expected that trees in heavily thinned stands would show higher radial growth rates than those in moderately thinned stands.

MATERIALS AND METHODS

Study site

The study was conducted in a 60 year old *Pinus halepensis* Mill. plantation ("Los Cuadros", 38º05' N, 1º06' W) located near the city of Murcia in SE Spain. Elevation in the experimental area ranges from 140 to 170 m above sea level. The climate is semiarid Mediterranean, with mean annual precipitation of 280 mm and average annual temperature of 18.2 ºC (data from the Spanish Agencia Estatal de Meteorología, Ministerio de Medio Ambiente, y Medio Rural y Marino). Potential annual evapotranspiration, calculated by the Thornthwaite method (e.g. Dunne & Leopold 1978), is 932 mm. Soils in the area are mostly haplic calcisols, with some lithic leptosols (Alías *et al.* 1998). The *Pinus halepensis* plantation was established on abandoned agricultural lands in the 1940s and 1950s, with an initial planting density of 1400 trees ha^{-1}. Tree growth is very poor, with heights ranging from 4 to 8 m and basal diameters ranging from 7 to 20 cm (2003 data).

The year 2005 was extremely dry in the experimental area, with a total annual precipitation of only 158.8 mm. Years 2004 and 2007 were moderately wet (324.7 mm and 363.6 mm annual precipitation, respectively) but had very different rain distributions: whereas 2004 had a rainy spring, 2007 had a dry spring and above-average rainfall during late summer and early fall (Fig. 1).

Experimental and sampling design

The forest thinning experiment is a randomized block design with one factor

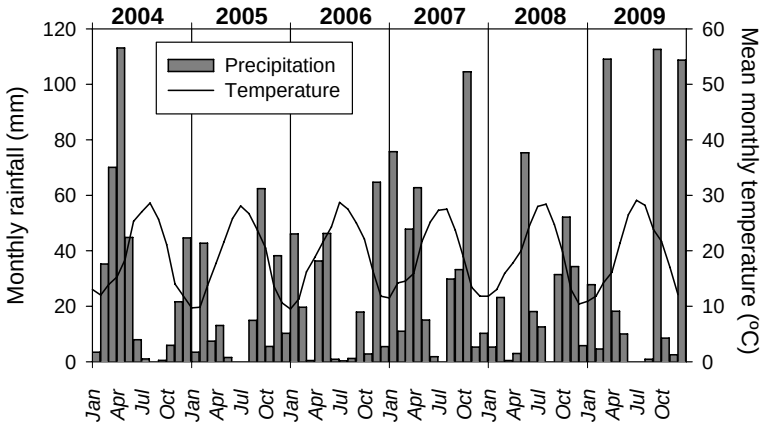

Figure 1. Monthly rainfall and mean monthly temperatures between January 2004 and December 2009 (data from Murcia weather station provided by the Spanish Agencia Estatal de Meteorología).

(thinning intensity) and three replicate blocks. All replicate blocks (60x120 m each) are located on moderately steep (less than 20%) slopes. One replicate block is located on a site with north-west (NW) aspect and the other two on sites with north-east (NE) aspect. Each replicate block was divided in two adjacent plots of 60x60 m each, and each plot was assigned one of two different thinning treatments: heavy thinning, with approximately 50% reduction of initial basal area, and a final tree basal area of 4.5 m^2/ha left standing; or moderate thinning, with approximately 33% reduction of initial basal area, and a final tree basal area of 6.6 m^2/ha left standing. Thinning treatments were applied between August and November 2004. After thinning, some of the slash was left on site. Final tree density was approx. 770 trees ha^{-1} in the moderate thinning treatment, vs. approx. 550 trees ha^{-1} in the heavy thinning treatment. The effects of thinning treatments on the carbon and water relations of the remaining trees were assessed within a 35x35 m core area in each experimental plot, which allowed for a 25 m wide buffer zone around the core area to avoid boundary or edge effects.

Bulk leaf material was collected in the fall of 2005 and 2007 for isotopic and elemental analyses. In November 2005, about one year after thinning, pine needle samples were collected from 6 randomly selected trees in each plot (6 trees x 2 treatments x 3 replicate blocks = 36). All sampled trees had diameters at breast height ranging from 10 to 20 cm. Current year needles (2005 leaf cohort) and needles formed during the previous year (2004 leaf cohort) were collected from south-facing branches in the upper portion of the tree crown, using a telescopic pruner. In November 2007, current year needles (2007 leaf cohort) and lignified stem sections were collected from 10 trees in each plot (10 trees x 2 treatments x 3 replicate blocks = 60 trees, including the same 36 trees sampled in 2005). New leaves of *Pinus halepensis* flush in spring and grow throughout the summer and into early fall (Dougherty, Whitehead & Vose 1994; Weinstein 1989). Foliar samples were taken in November, when all the pine needles were fully expanded. In May 2009, leaf gas exchange measurements (A, g_s, WUE_i) were conducted in order to validate the interpretation of isotopic data. Leaf gas exchange measurements were made on 9-12 remaining trees per treatment in each replicate block (~30 trees per thinning treatment overall). In October 2010, two increment cores were taken at ~ 1.30m height from each of the same 36 pines sampled in 2005, for tree ring growth analysis.

Leaf elongation, nutrient concentrations and isotopic composition

The length of ten randomly selected pine needles per sampled tree was measured with a precision of 1 mm for each leaf cohort (2004, 2005 and 2007). Bulk leaf samples were oven-dried at 60 ºC and ground to a fine powder using a ball mill. Finely ground bulk leaf samples were weighed using a precision balance, and encapsulated in tin capsules for

stable isotope analyses. Bulk leaf material contains varying proportions of organic compounds with different isotopic signatures; however, several studies measuring $\delta^{18}O$ and $\delta^{13}C$ in both bulk leaf material and leaf cellulose have found that the stable isotope compositions of these materials are strongly positively correlated (e.g. Barbour *et al.* 2000; Sullivan & Welker 2007; Powers *et al.* 2008; although cellulose extraction may be necessary when comparing plants grown under different environmental conditions, as in Grams *et al.* 2007).

The carbon isotope ratio of bulk leaf material ($\delta^{13}C$) was analysed using elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS). The oxygen isotope ratio of bulk leaf material ($\delta^{18}O$) was determined with a Finnigan MAT Delta Plus XL (Finnigan MAT, Bremen, Germany) following the method described in Farquhar, Henry & Styles (1997). This method uses a high purity alumina pyrolysis tube, a pyrolysis temperature of 1130ºC, chloropentane doping of the carrier gas, and a Porapak Q gas chromatography (GC) column before the molecular sieve column. The Porapak Q column separates N_2/CO_2 from any organic compounds, the N_2 and CO_2 is allowed to proceed to and be separated on the molecular sieve column, then both columns are back flushed to remove any contaminates before the next sample is analysed. Isotope analyses were conducted at the Center for Stable Isotope Biogeochemistry, University of California, Berkeley (USA). Isotope ratios are expressed in delta notation (‰), where the isotopic composition of a material relative to that of an internationally accepted reference standard is given by $\delta^{xx}E = (R_{sample}/R_{standard}-1) \times 1000$, where R is the molecular ratio of heavy to light isotope forms (Dawson *et al.* 2002). The standard used for $\delta^{13}C$ is PeeDee Belemnite (V-PDB) and the standard for $\delta^{18}O$ is Vienna-standard mean ocean water (V-SMOW). Long-term (3+ year) external precisions for carbon and oxygen isotope analyses are 0.14 and 0.23‰, respectively.

Foliar nitrogen and carbon concentrations were determined with a Thermo Finnigan Flash 1112 elemental analyzer (Franklin, MA, USA). Foliar phosphorus and potassium concentrations were measured at Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas (CEBAS-CSIC) by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Elemental Iris Intrepid II XDL, Franklin, USA) after a microwave-assisted digestion with HNO_3:H_2O_2 (4:1, v:v).

Stem water content and isotopic composition

In November 2007, lignified stem sections were collected from the same trees sampled for bulk leaf material. Lignified stem sections from remaining trees in both treatments were also collected in May 2008 (n = 24) and September 2008 (n = 14) in order to obtain stem water content and isotopic composition values representative of the growing season. Two stem sections per tree, approximately 10 mm in diameter and 20 mm long, were collected for stem water extractions, and all leaf and green stem tissue was removed from them to avoid contamination of xylem water with isotopically enriched water (Ehleringer & Dawson 1992). Upon collection, stem samples were immediately placed in capped vials, wrapped with Parafilm, and stored in the freezer until water extraction. Stem water was extracted using a cryogenic vacuum distillation line (Ehleringer, Roden & Dawson 2000). Stem water content was calculated from the weight difference before and after oven-drying stem samples at 100 ºC. In semiarid ecosystems, inter-plant differences in stem water content often reflect differences in plant water status (e.g. plant water potential; Querejeta, Egerton-Warburton & Allen 2009).

Analysis of stem water $\delta^{18}O$ ($\delta^{18}O_{stem\ water}$) was conducted at the Stable Isotope Laboratory of the Department of Earth and Planetary Sciences, University of New Mexico (USA), using the CO_2 equilibrium

technique. The water samples (1 ml each) were injected into borosilicate vials equipped with rubber septa, which were previously purged with He–CO_2 gas mixture (0.5% CO_2). After 24 h equilibration at 25 ºC, the CO_2 was measured by continuous flow isotope ratio mass spectrometry using an automated CombiPal—Gas Bench system coupled to a Finnigan Mat Delta Plus mass spectrometer. The results were corrected using three laboratory standards (calibrated against international water standards). Reproducibility was better than 0.1% based on repeats of laboratory standards. $\delta^{18}O$ values are expressed in delta notation (‰) relative to the international standard V-SMOW.

Since leaf $\delta^{18}O$ is strongly influenced by the isotopic signature of the source water used by the plant (Dawson *et al.* 2002; Barbour 2007), it is standard practice to report the oxygen isotopic composition of leaf tissue as oxygen isotope enrichment above that of source water, in order to account for this effect. Leaf oxygen isotope enrichment above source water ($\Delta^{18}O$; Barbour *et al.* 2000) is calculated as:

$$\Delta^{18}O = \text{bulk leaf } \delta^{18}O - \delta^{18}O_{\text{stem water}}$$

We used the $\delta^{18}O_{\text{stem water}}$ values measured in November 2007 to calculate leaf $\Delta^{18}O$, although it should be noted these $\delta^{18}O_{\text{stem water}}$ values may not be exactly representative of mean source water $\delta^{18}O$ integrated over the entire leaf growing season (spring to fall).

Leaf gas exchange measurements

Net photosynthetic rate (A) and stomatal conductance (g_s) were measured in May 2009 with a portable photosynthesis system (LI-6400, LICOR, Inc., Lincoln, NE, USA) equipped with a LI-6400-40 Leaf Chamber Fluorometer (LI-6400, LICOR, Inc., Lincoln, NE, USA) and a LICOR 6400-01 CO_2 injector. Gas exchange was measured on 1-year-old sunlit needles from apical shoots in southerly oriented branches in the middle part of the crown. Approximately twenty attached needles were placed in a 2 cm^2 leaf cuvette for gas exchange measurements. The CO_2 concentration in the cuvette was maintained at 380 μmol mol^{-1} CO_2. Measurements were done at saturating light of 1.500 μmol m^{-2} s^{-1}, and at ambient air temperature and relative humidity. The leaf-to-air water vapour pressure difference was between 0.75 and 1.2 mmol mol^{-1} for all measurements and the air flow was set to 350 μmol s^{-1}. All leaf gas exchange measurements were conducted at mid-morning between 9:00-11:00 h (local standard time; 7:00-9:00 GMT) on sunny days. Pine needles were collected after leaf gas exchange measurements, and the leaf sections enclosed in the leaf cuvette of the LI-COR 6400 were digitized by scanning on A3 flatbed scanner (HP Deskcan) fitted with a transparency adaptor at 300 dpi, using an 8-bit grayscale. We analyzed the images with specific software (WinRhizo, Regent Instruments Inc., Québec, Canada) to obtain needle surface area (and needle average diameter; Li, Kräuchi & Dobbertin 2006; Fuentes *et al.* 2007). Total needle surface area values measured by this method were on average 7.5% higher (2.15 cm^2) than the area of the leaf cuvette (2.00 cm^2). All gas exchange parameters were expressed on a total needle surface area basis. Intrinsic water-use efficiency (WUE_i) was calculated as A/g_s.

Tree ring growth analysis

Increment cores taken in October 2010 were mounted, air dried and fine-sanded until tree-rings were clearly visible under a binocular microscope. Tree rings were visually cross-dated and measured to the nearest 0.01mm with a measuring table (LINTAB) coupled with the TSAP software package (Rinn 1996). Obtained tree-ring series were compared with each other and with a previously constructed chronology for the same species in a neighbouring site (de Luis *et al.*, 2009) to ensure accuracy of cross-dating. Finally, cross-dating was statistically verified using the program COFECHA (Laboratory of Tree-Ring Research, University of Arizona, Tucson, AZ, USA; Holmes 1983). For each tree, ring widths of

Table 1. Levels of significance of main effects (*thinning intensity*, *replicate block, leaf cohort* and the interaction between *thinning intensity x leaf cohort*) on the leaf stable isotope composition, needle length and leaf nutrient concentrations of two leaf cohorts produced after thinning (2005 and 2007) in *Pinus halepensis*, as determined by repeated measures analysis of variance**.**

		df	$\delta^{13}C$	$\delta^{18}O$	Needle length	C	N	C/N	P	K
Between subjects effects	Thinning intensity	1	0.108	**0.004**	0.238	0.496	0.196	0.310	0.358	0.556
	Replicate block	2	0.311	**0.003**	0.322	0.663	**0.014**	**0.008**	0.051	**< 0.001**
Within subjects effects	Leaf cohort	1	**0.025**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.005**	0.269	0.171
	Leaf cohort x Thinning intensity	1	0.697	0.340	0.866	0.187	**0.034**	0.086	0.427	0.308

Values of $P < 0.05$ appear in bold.

years 2004 to 2009 were converted to standardized index values (TRW index) by dividing them by the average annual radial growth increment of that tree during the period 1983-2003 (pre-thinning reference period).

Statistical analyses

All statistical analyses were performed with SPSS software (version 17.0, SPSS Inc., Chicago). Repeated measures analysis of variance was used to evaluate the effects of thinning intensity, replicate block (between-subject factors), annual leaf cohort/year (within-subject factor) and their interactions on the dependent variables. Individual trees were considered subjects. The dependent variables were pine needle length, foliar nutrient concentrations and foliar stable isotopes ratios in the two annual leaf cohorts (2005, 2007) produced after application of the thinning treatments (2004) and the natural logarithm of the TRW index of years 2005 to 2009. Treatment effects on leaf gas exchange parameters, stem water content and stem water $\delta^{18}O$ (measured in a single year) were tested using two-way ANOVAs (considering thinning intensity as fixed factor and replicate block as random factor). Mean values ± standard error of measured variables in each treatment are presented. For each leaf cohort/year, linear regression analyses were used to examine the relationships between measured variables across all sampled individuals from both thinning treatments.

RESULTS

Foliar $\delta^{18}O$ and $\delta^{13}C$

Repeated measures analysis of variance indicates that thinning intensity significantly affected the foliar $\delta^{18}O$ of remaining trees (Table 1; Fig. 2a). Mean bulk leaf $\delta^{18}O$ and $\delta^{13}C$ in the 2004 leaf cohort (fully formed before thinning) were indistinguishable between trees that were to be assigned to different thinning treatments. However, a striking difference between treatments became apparent shortly (12 months) after thinning: foliar $\delta^{18}O$ in the 2005 leaf cohort (newly formed after thinning) was significantly less enriched in the heavily thinned than in the moderately thinned stands (Fig. 2a; P=0.010). This difference in foliar $\delta^{18}O$ between thinning treatments was still apparent in the 2007 leaf cohort (Fig. 2a; P=0.035). Interestingly, the difference in bulk leaf $\delta^{18}O$ between heavily and moderately thinned stands was largest for the 2005 leaf cohort, which was produced during the driest year of the study period (Fig. 2a).

In contrast to $\delta^{18}O$, bulk leaf $\delta^{13}C$ was unaffected by thinning intensity (Table 1; Fig. 2b). Foliar $\delta^{13}C$ in the 2007 leaf cohort (but not in the 2005 cohort) was positively correlated with foliar $\delta^{18}O$ across individuals from both thinning treatments (Fig. 3a).

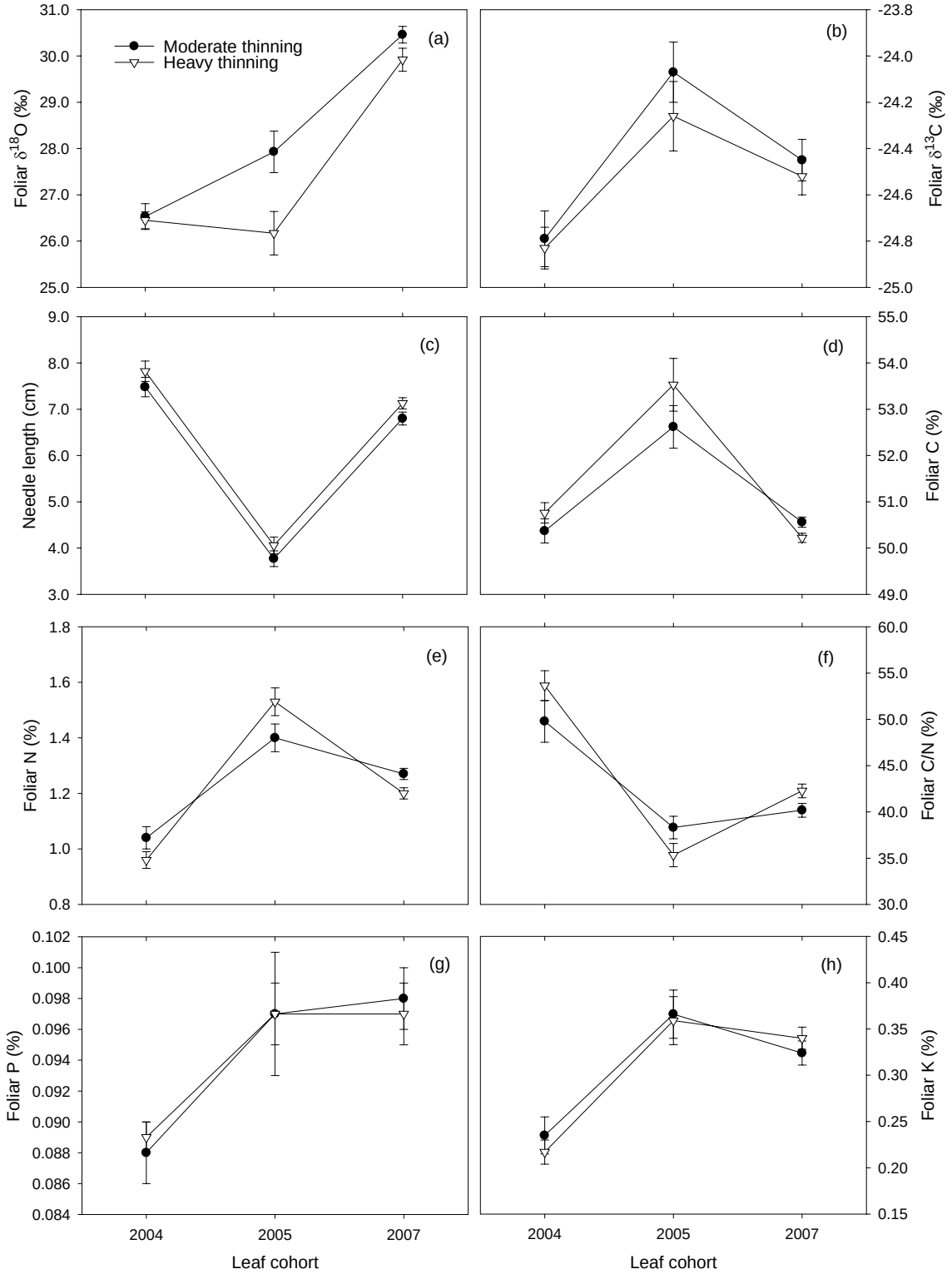

Figure 2. Mean values of *(a)* bulk leaf $\delta^{18}O$, *(b)* bulk leaf $\delta^{13}C$, *(c)* needle length, *(d)* leaf carbon concentration (C), *(e)* leaf nitrogen concentration (N), *(f)* leaf carbon to nitrogen ratio (C/N), *(g)* leaf phosphorus concentration (P) and *(h)* leaf potassium concentration (K) in remaining *Pinus halepensis* trees of the heavy and moderate thinning treatments for the 2004, 2005 and 2007 leaf cohorts (error bars indicate ± one SE). Thinning treatments were applied in the fall of 2004.

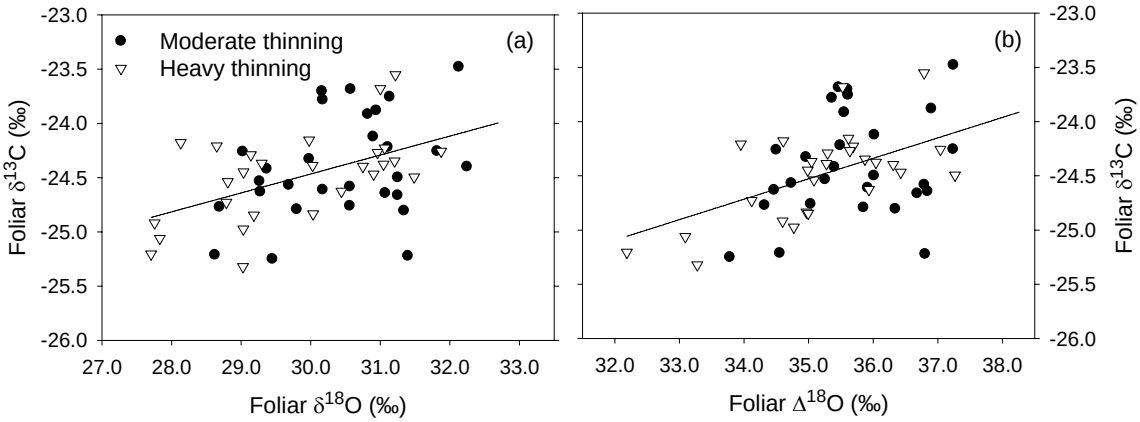

Figure 3. Relationships between (a) bulk leaf $\delta^{13}C$ and $\delta^{18}O$ ($\delta^{13}C = -29.70 + 0.17\delta^{18}O$; r = 0.46, P<0.01, $r^2 = 0.21$, n = 56), and (b) bulk leaf $\delta^{13}C$ and foliar oxygen isotope enrichment above source water ($\Delta^{18}O$) ($\delta^{13}C = -31.12 + 0.19\Delta^{18}O$; r = 0.46, P<0.01, $r^2 = 0.21$, n = 55) in the 2007 leaf cohort of remaining *Pinus halepensis* trees, across individuals from both thinning treatments.

Leaf growth and elemental concentrations

Repeated measures analysis of variance showed no significant difference in pine needle length between thinning treatments (Table 1). However, remaining pines in the heavily thinned stands tended to have somewhat longer needles than those in the moderately thinned stands, and this difference became marginally significant for the 2007 leaf cohort (P = 0.080), when average needle length was 7.1 ± 0.1 cm in heavily thinned stands vs. 6.8 ± 0.1 cm in moderately thinned stands (Fig. 2c). In both thinning treatments, pine needle length was much shorter in the 2005 cohort than in the other cohorts due to severe drought. In the 2005 leaf cohort, pine needle length was weakly negatively correlated with foliar $\delta^{18}O$ across individuals from both thinning treatments ($r^2 = 0.127$; P = 0.038).

Foliar N concentration was highest in 2005 for both thinning treatments, possibly due to a pulse of nitrogen availability derived from decomposition of dead fine roots following tree thinning. However, poor leaf growth due to severe drought may have also contributed to altered leaf stoichiometry and increased foliar N concentration in the 2005 leaf cohort. Foliar elemental concentrations in remaining trees were not significantly different between thinning treatments according to repeated measures ANOVA (Table 1; Figs. 2d-h). However, foliar N concentration became transiently higher in the heavily thinned than in the moderately thinned stands for the 2005 leaf cohort (produced shortly after thinning), whereas the reverse pattern was found in the 2004 (pre-thinning) and 2007 leaf cohorts.

Stem water content and isotopic composition

The $\delta^{18}O$ of stem water in remaining trees did not differ between moderately and heavily thinned stands in November 2007 (-5.38 ± 0.11‰ and -5.28 ± 0.10‰, respectively; P=0.459), May 2008 (-3.57 ± 0.08‰ and -3.39 ± 0.15‰, respectively; P=0.366) or September 2008 (-2.61 ± 0.32‰ and -2.20 ± 0.36‰, respectively; P=0.503). This indicates that the sources of water used by the remaining pines were indistinguishable between the two thinning treatments. There were no significant differences in stem water content between moderately and heavily thinned stands in November 2007 (51.36 ± 0.49% and 52.00 ± 0.39%, respectively; P=0.289) or May 2008 (49.30 ± 0.43% and 49.97 ± 0.58, respectively; P=0.354). However, mean stem water content was significantly higher in heavily than in moderately thinned stands (49.68 ± 0.68%

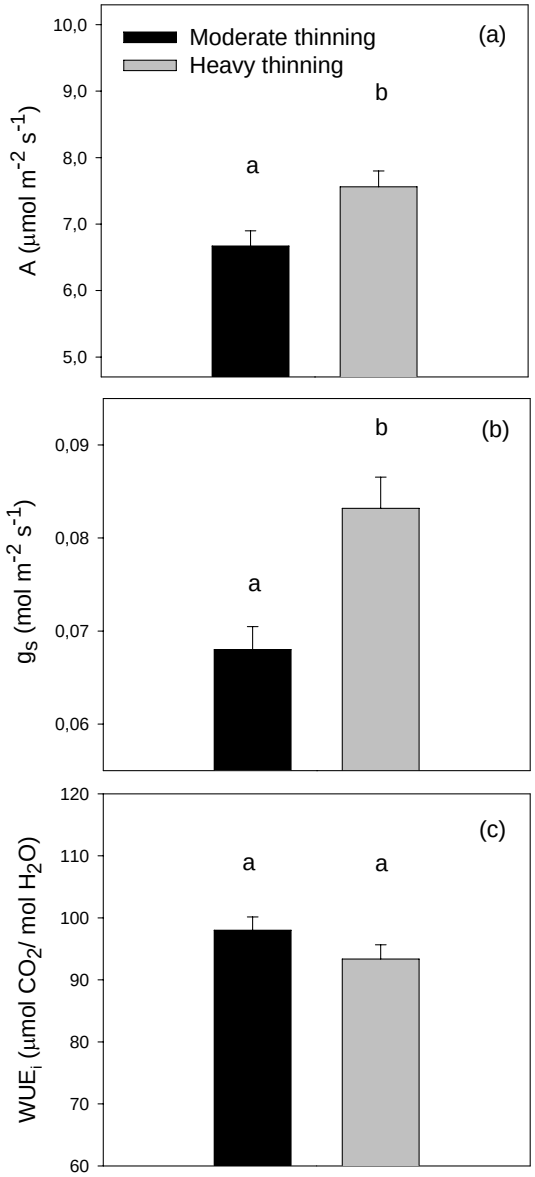

Figure 4. Effects of thinning intensity on the net photosynthetic rate (A), stomatal conductance (g_s) and intrinsic water use efficiency (WUE_i) of remaining *Pinus halepensis* trees. Different letters above columns indicate significant differences between thinning treatments ($P < 0.05$); error bars represent one SE.

vs. 46.45 ± 1.03% respectively; P<0.01) in late summer (September 2008).

Oxygen isotope enrichment of bulk leaf material above source water ($\Delta^{18}O$) was not significantly different between thinning treatments in November 2007 (35.32 ± 0.24‰ in heavily thinned stands vs. 35.69 ± 0.17‰ in moderately thinned stands;

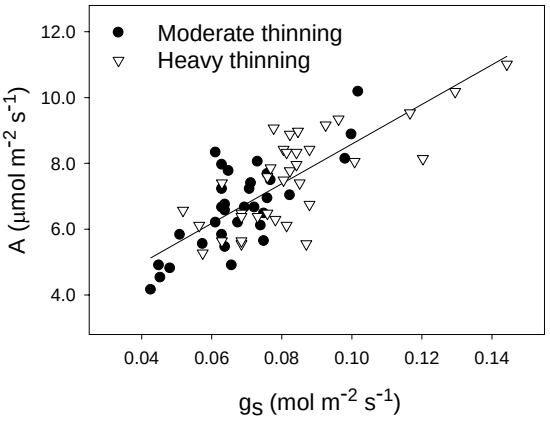

Figure 5. Relationship between net photosynthetic rate (A) and stomatal conductance (g_s) in remaining *Pinus halepensis* trees across individuals from both thinning treatments ($A = 2.56 + 60.18g_s$; $r = 0.78$, $P < 0.01$, $r^2 = 0.61$, $n = 68$). The A/g_s regression equation for the heavy thinning treatment was $A = 2.97 + 55.07g_s$ ($r^2 = 0.57$; $P < 0.01$, $n = 35$) vs. $A = 1.77 + 72.10g_s$ ($r^2 = 0.59$; $P < 0.01$, $n = 33$) for the moderate thinning treatment.

P=0.168). Across individuals from both thinning treatments, foliar $\Delta^{18}O$ was positively correlated with foliar $\delta^{13}C$ (Fig. 3b), whereas stem water content was weakly negatively correlated with bulk leaf $\delta^{18}O$ ($r^2 = 0.204$; $P < 0.001$) and $\Delta^{18}O$ ($r^2 = 0.142$; $P = 0.004$). Stem water content was not correlated with stem water $\delta^{18}O$.

Leaf gas exchange measurements

Net photosynthetic rate (A) and stomatal conductance (g_s) in remaining trees were both significantly higher in heavily thinned stands than in moderately thinned stands ($P = 0.008$ and $P = 0.001$, respectively; Fig. 4a-b). However, intrinsic water use efficiency (WUE_i) was not significantly different between thinning treatments (Fig. 4c), thus suggesting roughly parallel increments of similar magnitude in both A and g_s after heavy thinning. There was a strong positive correlation between A and g_s across individuals from the two thinning treatments (Fig. 5), thus indicating tight stomatal control of both transpiration and carbon assimilation rate in *Pinus halepensis*.

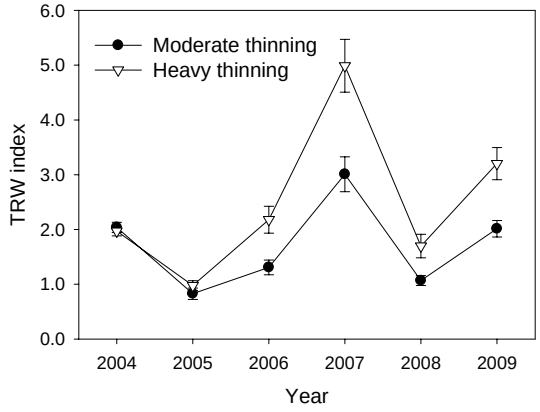

Figure 6. Mean tree ring width index (TRWindex) of *Pinus halepensis* trees in the heavily and moderately thinned stands from 2004 to 2009 (n = 18; error bars indicate ± one SE). Thinning treatments were applied in the fall of 2004. TRW index is calculated by dividing each annual tree-ring width by the average annual radial growth increment of that tree during the period 1983-2003 (pre-thinning reference period).

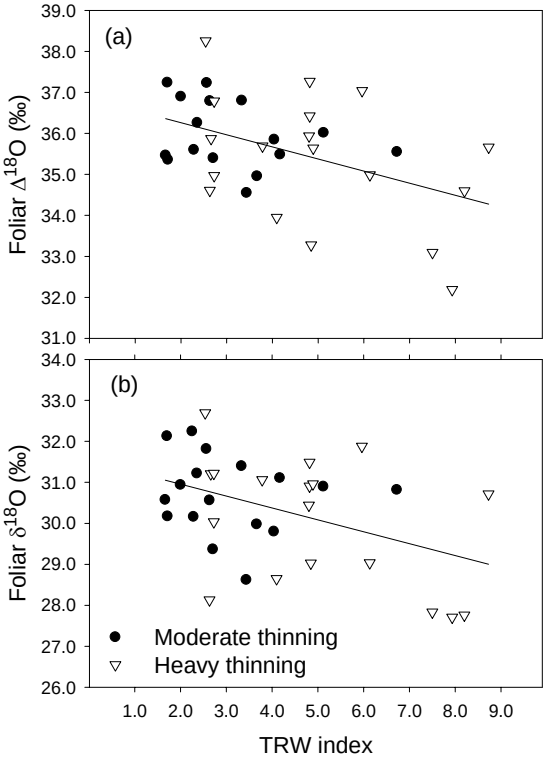

Figure 7. Relationship of tree ring width index (TRWindex) in 2007 with (a) foliar oxygen isotope enrichment above source water of the 2007 leaf cohort ($\Delta^{18}O$; r = -0.45, P < 0.01, r^2 = 0.20, n = 34; $\Delta^{18}O$ = 36.85 – 0.29TRWindex) and (b) bulk leaf $\delta^{18}O$ in the 2007 leaf cohort (r = -0.43, P = 0.01, r^2 = 0.18, n = 35, $\delta^{18}O$ = 31.54 – 0.29TRWindex) across individuals from both thinning treatments.

Tree ring growth

Repeated measures analysis of variance indicates that remaining trees in heavily thinned stands had significantly greater radial growth than those in moderately thinned stands during the period 2005 to 2009 (Fig. 6; P<0.001). Mean annual tree ring widths (TRW) from 2005 to 2009 were 1.64 ± 0.12mm in heavily thinned stands vs. 1.14± 0.11mm in moderately thinned stands. For the 1983-2003 period (before thinning) there were no significant differences in TRW between stands assigned to different thinning intensities (mean annual TRW for this period is 0.66 ± 0.04mm in plots assigned to heavy thinning vs. 0.71 ± 0.06mm in plots assigned to moderate thinning; repeated measures analysis of variance, P=0.520). Although the thinning treatments were applied in the fall of 2004, significant differences in TRW index between thinning intensities did not appear until 2006 (Fig. 6).

In 2007, TRW index was negatively correlated with foliar $\Delta^{18}O$ (Fig. 7a) and foliar $\delta^{18}O$ (Fig. 7b) across individuals from both thinning treatments. TRW index was also significantly correlated with foliar $\delta^{13}C$ (r = -0.44, P=0.01, r^2 = 0.20) and stem water content (r = 0.45, P<0.01, r^2 = 0.20), but not with stem water $\delta^{18}O$.

DISCUSSION

Differences in the foliar $\delta^{18}O$ of remaining trees between heavily and moderately thinned stands cannot be plausibly explained in terms of differences in microclimatic conditions. Reducing tree density opens the canopy and changes microclimatic conditions by allowing greater penetration of solar radiation and wind into forest stands (Brèda *et al.* 1995; Aussenac 2000). Thinning leads to significant changes in light availability,

temperature and humidity in the forest environment, and these changes in microclimatic conditions could affect the foliar $\delta^{18}O$ of remaining trees (Yakir 1992; Barbour 2007). Forest sites with more open canopies generally show greater light intensity, wider temperature fluctuations and lower air relative humidity than neighbouring sites with more closed canopies (Aussenac 2000). In water-limited conifer forests, low-density stands with more open canopies are usually sunnier, hotter and drier than neighbouring stands with denser canopies (Meyer *et al.* 2001; Rambo & North 2009; Ma *et al.* 2010; Martín-Benito *et al.* 2010). Higher temperature and lower air humidity (higher vapour pressure deficit, VPD) would be expected to lead to more enriched foliar $\delta^{18}O$ in semiarid forest with more open canopies (Burk & Stuiver 1981; Yakir 1992; Roden & Ehleringer 1999; Barbour 2007). However, we found that remaining trees in low-density stands (heavily thinned) with more open canopies showed consistently lower foliar $\delta^{18}O$ values than those in moderate-density stands (moderately thinned) with denser canopies.

Lower foliar $\delta^{18}O$ in heavily thinned stands supports our prediction that a greater reduction in inter-tree competition for soil resources would be more effective at enhancing the water status of remaining trees (Fig. 2a). *Pinus halepensis* is a drought-avoiding species with tight stomatal control of transpiration and photosynthesis under water-limiting conditions (Ferrio *et al.* 2003; Klein *et al.* 2005; Maseyk *et al.* 2008; Voltas *et al.* 2008). Lower foliar $\delta^{18}O$ suggests higher leaf-level stomatal conductance in stands subjected to heavy thinning than in stands subjected to moderate thinning (Barbour *et al.* 2000; Barbour 2007; Farquhar *et al.* 2007), and this interpretation of isotopic data is well supported by leaf gas exchange data (Fig. 4b). As expected, a greater decrease in canopy interception of rainfall and inter-tree competition for soil water led to higher stomatal conductance in the remaining trees of heavily thinned stands (Brèda *et al.* 1995). Whereas foliar $\delta^{18}O$ in the 2004 leaf cohort (produced before thinning) was indistinguishable between plots assigned to different thinning treatments, significant differences were apparent in the 2005 leaf cohort (produced after thinning, Fig. 2a), thus indicating a short response time (≈12 months) of bulk leaf $\delta^{18}O$ to differences in competition intensity in *Pinus halepensis*. Higher stomatal conductance in the remaining trees of heavily thinned stands apparently overwhelmed any potential effects of drier and warmer microclimatic conditions on foliar $\delta^{18}O$.

Differences in bulk leaf $\delta^{18}O$ between treatments were largest in a severe drought year (2005, with annual rainfall of only 159 mm; Fig 2a). Intense drought likely aggravates the negative effects of inter-tree competition on tree water status in semiarid forests, so heavy thinning may have been particularly beneficial to the water relations of remaining trees during a very dry year. The difference in foliar $\delta^{18}O$ between heavily and moderately thinned stands was less pronounced (but still significant) in a relatively wet year (2007, 364 mm annual rainfall), when inter-tree competition for soil water may have been less intense.

Stem water $\delta^{18}O$ was unaffected by forest stand density, thus indicating that the source water used by the remaining *Pinus halepensis* trees was indistinguishable between thinning treatments. Stem water $\delta^{18}O$ reflects the isotopic signature of the soil water sources used by the plant, as no fractionation occurs during water uptake by plant roots (Dawson & Ehleringer 1991; Barbour 2007; Ellsworth & Williams 2007). Since pines used soil water of nearly identical $\delta^{18}O$ in heavily and moderately thinned stands, the possibility that differences in the isotopic signature of source water might have been responsible for differences in bulk leaf $\delta^{18}O$ between thinning treatments (Barbour 2007) can probably be ruled out.

Foliar $\delta^{13}C$ did not differ between thinning treatments, thus suggesting that time-integrated intrinsic water use efficiency in the remaining trees was unaffected by forest stand density. This interpretation is well supported by leaf gas exchange data showing no difference in intrinsic water use efficiency ($WUE_i = A/g_s$) between heavily

and moderately thinned stands despite significantly higher A and g_s in the former (Fig. 4). Lack of response of foliar $\delta^{13}C$ to forest thinning in spite of enhanced A and g_s in remaining trees was reported by Sala *et al.* (2005) and McDowell *et al.* (2006), who attributed this result to parallel increases of similar magnitude in both parameters. According to the semi-quantitative dual isotope models developed by Scheidegger *et al.* (2000) and Grams *et al.* (2007), similar foliar $\delta^{13}C$ combined with lower foliar $\delta^{18}O$ in heavily than in moderately thinned stands must be interpreted as indicating higher g_s and A in the former. Once again, this interpretation of isotopic data is in good agreement with leaf gas exchange data (Fig. 4). Further, higher carbon assimilation rate translated into greater radial growth of the remaining trees in heavily thinned stands than in moderately thinned stands (Fig. 6). Interestingly, bulk leaf $\delta^{18}O$ responded to heavy thinning much earlier (Fig. 2a) than tree radial growth.

Foliar $\delta^{13}C$ was positively correlated with both leaf $\delta^{18}O$ and $\Delta^{18}O$ in the 2007 leaf cohort, thus suggesting that $\delta^{13}C$ is (at least partly) under stomatal control in *Pinus halepensis* (Scheidegger *et al.* 2000; Barbour, Walcroft & Farquhar 2002; Keitel *et al.* 2003; Voltas *et al.* 2008). The strong positive correlation between photosynthetic rate and stomatal conductance (Fig. 5) and the negative relationships of TRW index with $\Delta^{18}O$ and $\delta^{18}O$ in 2007 (Fig. 7) across individuals from both thinning treatments further indicate that carbon assimilation rate in *Pinus halepensis* is largely regulated by changes in stomatal aperture in response to soil water availability. Thus, our data suggest that higher photosynthetic rates in the remaining pines of heavily thinned stands resulted primarily from higher stomatal conductance (and therefore lower stomatal limitation to carbon fixation), as compared to moderately thinned stands. However, possible differences in light availability and/or in the biochemical photosynthetic capacity of leaves between thinning treatments may have also played a role (Martín-Benito *et al.* 2010). The photosynthetic capacity of leaves is positively related to plant nutrient status and foliar N concentration because it depends on the concentration of N-containing enzymes, pigments and electron transporters in leaf tissue (Reich *et al.* 1995; Dawson *et al.* 2002). In 2005, the foliar N concentration reached the highest values in both treatments (Fig. 2e), probably as a result of impaired needle growth during this drought year. However, foliar N was transiently higher in heavily than in moderately thinned stands in 2005 (Fig. 2e); since there was not significant difference in mean needle length between treatments, this may have been the result of a larger "pulse" of nitrogen availability in soil shortly after heavy thinning. Higher carbon assimilation rate in heavily thinned stands may thus have been partly attributable to enhanced biochemical photosynthetic capacity of pine needles due to improved plant N status.

In conclusion, heavy thinning reduced inter-tree competition for water more effectively than moderate thinning, thus resulting in higher stomatal conductance, photosynthetic activity and radial growth in the remaining trees. Simultaneous measurement of $\delta^{18}O$ and $\delta^{13}C$ in bulk leaf material helped characterize the leaf-level physiological response of the remaining trees to different thinning intensities at an early stage (≈ 12 months after stand density manipulation). To the best of our knowledge, this is the first study reporting a significant response of foliar $\delta^{18}O$ to silvicultural thinning intensity. Our results show that the dual isotope approach is particularly useful in situations where foliar $\delta^{13}C$ is unaffected by thinning treatments due to parallel increases of similar magnitude in both photosynthetic activity and stomatal conductance. Bulk leaf $\delta^{18}O$ has a short response time to thinning, which may be an important consideration for managers interested in assessing the effectiveness of silvicultural practices in low productivity forests where tree radial growth responses may take longer to become detectable.

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CAPÍTULO 5

AFFORESTATION WITH *PINUS HALEPENSIS* REDUCES THE LONG-TERM PHYSIOLOGICAL PERFORMANCE AND GROWTH OF THE DOMINANT NATIVE SHRUB *RHAMNUS LYCIOIDES* IN A MEDITERRANEAN ECOSYSTEM

Afforestation with *Pinus halepensis* reduces the long-term physiological performance and growth of the dominant native shrub *Rhamnus lycioides* in a Mediterranean ecosystem

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ABSTRACT

We performed a retrospective comparison (1989-2007) of growth ring widths, $\delta^{13}C$ and $\delta^{18}O$ of *R. lycioides* shrubs growing within *P. halepensis* plantations vs. shrubs growing in neighbouring open woodlands. We also measured leaf $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$ and foliar nutrient concentrations of *R. lycioides* shrubs growing at different distances from the nearest *P. halepensis* tree in dense afforested plantations. Results suggested strong inter-specific competition for water and nutrients between *P. halepensis* and *R. lyciodies*. *R. lycioides* shrubs growing in the afforested plantation showed higher $\delta^{13}C$ values of wood and lower radial growth than those in open woodlands, indicating lower stomatal conductance and photosynthetic activity of shrubs in the former stand type. Within the afforested stands, *R. lycioides* shrubs growing closer to the nearest *P. halepensis* tree were forced to rely on more ephemeral and superficial soil water, which reduced their stomatal conductance (higher leaf $\delta^{18}O$ values) and interfered with nutrient uptake (lower foliar N and P concentrations and more negative leaf $\delta^{15}N$). The intense competition for soil resources between *P. halepensis* and *R. lycioides* was more important in wet years, while during dry years all shrubs were severely water stressed irrespective of the competition intensity with *P. halepensis*.

Key words: *Pinus halepensis*, *Rhamnus lycioides*, competition, intrinsic water use efficiency, carbon isotopic composition, radial growth rings.

INTRODUCTION

Plant-plant interactions are key forces structuring vegetation assemblages and determining the composition of plant communities (Fowler 1986, Goldberg & Novoplansky 1997). Competition and facilitation are widespread in nature and can occur simultaneously, giving rise to complex interactions that may have variable outcomes depending among other factors on plants life stage and density, the severity of climatic conditions and indirect interactions with other species (Callaway & Walker 1997).

In semiarid environments, belowground competition for strongly limiting resources like water and nutrients is especially important (Fowler 1986). Alteration of the soil microbial and chemical environment by plant species may also play a fundamental role in plat-plant interactions (Callaway & Ridenour 2004, Klironomos 2002, Schenk 2006). According to the stress gradient hypothesis (Bertness & Callaway 1994), there may be a shift towards increasing frequency of positive plant-plant interactions in environments with severe abiotic stress. In water-limited environments, facilitative interactions involving water may occur through hydraulic lift and canopy shading (Filella & Peñuelas 2003a, Maestre *et al.* 2009, Prieto, Armas & Pugnaire 2012). Other facilitative interactions include positive impacts of plant species on soil nutrient availability (Temperton *et al.* 2007) and resource sharing through common mycorrhizal networks (Brooker *et al.* 2008). The importance of facilitative processes can prevail over the negative effects of competition in semiarid environments (e.g. Pugnaire, Armas & Valladares 2004). But the complexity of plant interactions is high and biotic interactions can shift from facilitation to competition within the same plant community due to variations in water availability across years (Tielbörger & Kadmon 2000) which can be very extreme in arid and semiarid regions (Fowler 1986). The level of resources availability determines the outcome of plant-plant interactions as was found by many studies along stress gradients (e.g. Cavieres *et al.* 2006, Pugnaire & Luque 2001). The outcome of plant-plant interactions can also change along the different life stages of plant species (Brooker *et al.* 2008, Miriti 2006, Pugnaire *et al.* 1996, Schiffers & Tielbörger 2006).

As a consequence, there is a need to understand trends in the net balance between positive and negative plant-plant interactions over long time periods that cover different climatic conditions (and their associated resources availability), and during different plant life stages (Butterfield *et al.* 2010, Cavard *et al.* 2011, McCluney *et al.* 2012). In addition, a deeper understanding of which particular response variables are most strongly affected by plant-plant interactions (e.g. survival, growth, physiological status…) can help to formulate better predictions about future shifts in plant community composition and structure under changing environmental conditions.

The carbon and oxygen isotopic composition of leaf material ($\delta^{13}C$ and $\delta^{18}O$, respectively) provides time-integrated information on leaf gas exchange processes during the growing season. When measured on radial growth rings, $\delta^{13}C$ and $\delta^{18}O$ provide long-term records of plant physiological processes. In C_3 plants, $\delta^{13}C$ is a good proxy of leaf-level intrinsic water use efficiency (WUE_i), which is given by the ratio between leaf net photosynthetic rate (A) and stomatal conductance (Dawson *et al.* 2002, Farquhar, Ehleringer & Hubick 1989). Plant $\delta^{18}O$ is influenced by source water $\delta^{18}O$, but is also inversely related to the ratio of atmospheric to leaf intercellular water vapour pressure (e_a/e_i), and can thus provide a time-integrated indication of leaf stomatal conductance (g_s) during the growing season (Barbour 2007, Farquhar, Cernusak & Barnes 2007). Measuring plant $\delta^{18}O$ can thus help to separate the independent effects of A and g_s on $\delta^{13}C$ (Grams *et al.* 2007, Moreno-Gutiérrez *et al.* 2011, Roden & Farquhar 2012, Scheidegger *et al.* 2000). On the other hand, the oxygen stable isotope composition of plant xylem water accurately reflects the isotope ratio of soil water used by plants, as no isotopic fractionation occurs during soil water uptake by roots (Barbour 2007).

The nitrogen isotope composition of plant material ($\delta^{15}N$) is related to nutrients and water availability (Tilman 1988). Several authors have found positive relationships between leaf $\delta^{15}N$ composition and leaf N concentration in non N_2-fixing plant species at local, regional and global scales and within and across species (Bai *et al.* 2009, BassiriRad *et al.* 2003, Craine *et al.* 2009, Hobbie, Macko & Williams 2000). But the interpretation of $\delta^{15}N$ is not straightforward, as it can be influenced by a combination of processes (utilization of different N-sources, mycorrhizal associations, spatial and temporal variations in N availability and demand, among others; (Bai *et al.* 2009,

Dawson *et al.* 2002, Högberg 1997). However, under appropriate conditions, plant $\delta^{15}N$ can provide valuable information and clues about some processes. For example, Bai *et al.* (2009) related leaf $\delta^{15}N$ of two evergreen shrubs with an index of proximity to a tree legume (*Prosopis glandulosa*) and found that leaf $\delta^{15}N$ values were closer to 0‰ near the N_2 fixing tree, thus indicating that the tree legume influenced the N nutrition of the non N_2-fixing evergreen shrubs. In another example, Filella & Peñuelas (2003b) found N partitioning among coexisting plant species in a Mediterranean environment based on inter-specific differences in their leaf $\delta^{15}N$.

Some studies have measured the stable isotope composition of tree rings to assess the long-term effects of intra-specific competition on tree performance (Linares *et al.* 2009, Moreno-Gutiérrez *et al.* 2012a). Other studies have assessed the long-term effect of thinning on the physiological status of remaining trees (Martín-Benito *et al.* 2010, McDowell *et al.* 2003). In a closer approach, Battipaglia *et al.* (2009) investigated differences in drought resistance among coexisting plant species by measuring the stable isotope composition of tree rings of *Picea abies* and *Abies alba*. However, no study up to date has used long-term records of $\delta^{13}C$ and $\delta^{18}O$ in growth rings to disentangle the processes underlying the outcome of inter-specific interactions.

This is especially important in the light of climate change, as the perception of environmental severity can be species-specific (Brooker *et al.* 2008) and thus, environmental changes can produce drifts in the outcome of plant-plant interactions which can determine the success of plant species. In this respect, semiarid Mediterranean areas are especially vulnerable to the predicted increases in temperature and decreases in precipitation (Machado *et al.* 2011, Millennium-Ecosystem-Assessment 2005) which could produce dramatic changes in the structure and composition of plant communities. The afforested plantations of *Pinus halepensis* Mill. that were extensively planted in semiarid areas of the Iberian Peninsula during the second half of the XX^{th} century are particularly susceptible to climate change and drought intensification (Maestre & Cortina 2004). They were initially planted in order to prevent erosion and facilitate subsequent succession to natural plant communities (Cortina *et al.* 2011, Maestre & Cortina 2004). However, several recent studies have found little evidence of re-colonization of *P. halepensis* plantations by late-successional woody species (Bellot *et al.* 2004, Cortina *et al.* 2011, Gómez-Aparicio *et al.* 2009, Maestre & Cortina 2004).

The aim of this study was to assess the long-term outcome of plant-plant interactions between *P. halepensis* planted trees and the dominant native shrub *Rhamnus lycioides* L., and to understand the physiological processes underlying the outcome of this interaction. In order to do so, we compared the growth rings widths (GRW) and oxygen and carbon isotopic compositions ($\delta^{13}C_R$ and $\delta^{18}O_R$, respectively) of *R. lycioides* shrubs growing in a dense afforested plantation of *P. halepensis* trees vs. those of shrubs growing in a nearby open woodland with widely scattered *P. halepensis* trees. Within the afforested plantation, we also measured leaf isotopic composition (C, O, N) and nutrient concentration, as well as stem water content and isotopic composition in *R. lycioides* shrubs growing at varying distances from the nearest pine, in two years with contrasting rainfall. We hypothesized that competition with *P. halepensis* trees would exert a negative effect on the long-term water and nutrient status of *R. lycioides* shrubs in the afforested plantation. However, we also hypothesized that facilitative interactions would prevail in drought years due to the beneficial effects of canopy shading on shrub water relations.

MATERIALS AND METHODS

Study sites

The experimental area comprises two *Pinus halepensis* stand types with sharply contrasting structure and Enhanced Vegetation Index (EVI) values (which are related to plant community transpiration, see

Moreno-Gutiérrez *et al.* 2012a): a dense 60 year old *Pinus halepensis* Mill. plantation (with a density of approximately 1150 trees ha^{-1}) and a nearby open woodland (with scattered *P. halpensis* trees and a density of less than 20 trees ha^{-1}). The climate is semiarid Mediterranean, with mean annual precipitation of 288 mm and an average annual temperature of 19ºC. The area is characterized by low hills (140-170m asl., < 20 % slopes) and soils are mostly haplic calcisols, with some lithic leptosols (according to FAO classification).

Dendroecological analysis

In May 2008, 10 individuals of *Rhamnus lycioides* L. were sampled in each stand type (20 in total). Stem sections from the main trunk were collected at ground height. Dating was difficult as is commonly found in Mediterranean plant species (Cherubini *et al.* 2003) and it was possible only in 8 individuals from the open woodland and 7 from the afforested plantation. From each stem cross section, annual growth rings were measured along three radii with a precision of 0.01 mm using a measuring table (LINTAB, Frank Rinn, Heidelberg, Germany) coupled with the TSAP software package (Frank Rinn, Heidelberg, Germany; (Rinn 1996). Cross-dating was statistically verified using the programs TSAP (by the Gleichläufigkeit, GLK: percentage of slopes intervals with equal sign in two time series) and COFECHA (Holmes 1983). Single-radii ring-width series were cross-dated with the mean of all individual growth series from the same stand type. GLK values were always significant ($P < 0.05$) and higher than 60%. In each stand type, individual growth series were detrended with ARSTAN (Cook & Holmes 1984, Holmes 2001) using a cubic smoothing spline with a 50% frequency response over 25 years and stabilized variance ("Briffa/Osborn" variance adjusted version, computed in ARSTAN; Osborn, Briffa & Jones 1997). Afterwards, an autoregressive model was applied to remove the autocorrelation with the previous year ring width. Individual series within each stand type were averaged with a robust (bi-weight) estimation of the mean (Cook 1985). The following parameters were calculated within each stand type: EPS (*Expressed Population Signal*; indicates the level of coherence of the constructed chronology and how it portrays the hypothetical perfect population chronology), r-bar (mean correlation among all possible pairings of individual series within a chronology) and *mean sensitivity* (indicates the degree to which annual ring width changes from year to year and how it is influenced by high-frequency climatic variations). Standardized residual values (GRW_{res}) were used for assessing correlations with climatic data.

Table 1. Mean shrub age (years) and dendrochronological characteristics of the residual mean chronology (calculated with ARSTAN, Holmes 2001) of *Rhamnus lycioides* in open woodland and afforested stands.

Characteristics	Open woodland	Afforested plantation
Mean shrub age and SE in 2007	49.4 (3.3)	41.3 (3.3)
Mean growth ring width and SE (mm) 1983-2007	0.56 (0.03)	0.35 (0.03)
Mean EPS (residual)	0.955	0.773
Mean R-bar (residual)	0.513	0.287
Mean sensitivity (residual)	0.299	0.282
Standard deviation (residual)	0.296	0.272
1st order partial autocorrelation (residual)	0.110	0.112

In 5 individuals per stand type, α-cellulose was extracted separately from each annual growth ring formed between 1989 and 2007 in order to analyze its stable isotope composition. The α-cellulose was extracted with a double step digestion, with a 5% NaOH solution at 60ºC for 2 hours followed by a 7% $NaClO_2$ + acetic acid solution at 60ºC for a minimum of 36 hours (Battipaglia *et al.* 2008, Boettger *et al.* 2007, Rinne *et al.* 2005).

Isotopic analyses of cellulose were conducted at the Instituto Andaluz de Ciencias de la Tierra on a Thermo Finnigan Delta Plus XL mass spectrometer. Two internal standards were used for analysis after every ten leaf samples: cellulose ($\delta^{13}C$ = -24.72‰) and phthalic acid ($\delta^{13}C$ = -30.63‰). The repeated analysis of these standards yielded an SD of less than 0.1‰. The isotopic composition of carbon is expressed in delta notation (‰) relative to the reference standard VPDB.

Annual intrinsic water use efficiency values (WUE_i) were calculated from the carbon isotopic composition of individual radial growth rings ($\delta^{13}C_R$) using the model described Farquhar *et al*. (1982):

$$(1) \qquad WUE_i = \frac{c_a(b - \Delta^{13}C)}{1.6(b-a)}$$

with

$$\Delta^{13}C = \frac{(\delta^{13}C_{atm} - \delta^{13}C_R)}{(1+\delta^{13}C_R)}$$

where $\Delta^{13}C$ is the photosynthetic discrimination against ^{13}C in the atmosphere (‰), $\delta^{13}C_{atm}$ is the carbon isotope composition of atmospheric CO_2, c_a is the mean annual atmospheric CO_2 concentration, a is the fractionation during CO_2 diffusion through stomata (4.4‰), and b is the fractionation during carboxylation (27‰). Annual values of c_a and $\delta^{13}C_{atm}$ were obtained from Francey *et al.* (1999), McCarroll *et al.* (2009) and McCarroll & Loader (2004). The calculation of WUE_i values removes the effects of the decline in the $\delta^{13}C$ of atmospheric CO_2 and the increase in atmospheric CO_2 concentration due to fossil fuel emissions on the carbon isotope ratios of annual growth rings. Thus, WUE_i values were used in all the statistical analyses.

Meteorological data

Meteorological data were provided by the Spanish National Meteorological Agency (AEMET). Monthly values of mean temperature and precipitation from 1983 to 2007 were obtained from the "Embalse de Santomera" meteorological station (38º05' N, 1º05' W, 90 masl), located near the sampling sites (<5 km). Missing data were obtained by simple linear regression with the nearby meteorological stations of "Santomera" (38º03' N, 1º02' W, 36 masl) and "Murcia-Alfonso X" (37º59' N, 1º07' W, 90 masl). Atmospheric vapor pressure deficit (VPD) was calculated using the model of Ferrio & Voltas (2005) for the Mediterranean region. From monthly meteorological data we calculated seasonal values (three months periods: January to March, April to June, July to September, October to December), annual values (January to December) and values for the hydrological year (from October of the previous year to September of the current year, oct-Sept) of every measured variable (T, P, VPD).

Inter-species competition analysis

Within the *P. halepensis* plantation, *R. lycioides* shrubs growing at varying distances (10-300 cm) from the nearest pine were sampled at the end of May in two years with contrasting meteorological conditions: 2007 (n = 13 ; total precipitation amount from March to May = 125.5 mm) and 2008 (n = 18 ; total precipitation amount from March to May = 78.7 mm). Distance to the nearest pine was recorded as a measure of competition intensity between *R. lycioides* and *P. halepensis*. In May 2008, the Hegyi's competition index (HCI; Hegyi, 1974) was also calculated using the diameter at breast height (DBH) and the distance of all trees within 3 m from each target shrub and the

external diameter of each shrub. However, HCI and distance to the nearest pine showed similar relationships with the analyzed plant variables, so we decided to use distance to the nearest pine as indicator of competition intensity in order to maintain consistency with data from May 2007.

In both sampling campaigns, fully sun-oriented leaves and twig sections (approximately 10 mm in diameter and 20 mm long) were collected from adult shrubs for stable isotopes analyses. After collection, lignified twig sections were immediately placed in capped vials, wrapped with Parafilm, and stored in the freezer until water extraction with a cryogenic vacuum distillation line (Ehleringer, Roden & Dawson 2000). Stem water content was calculated gravimetrically. Bulk leaf samples were oven-dried at 60 ºC, finely ground with a ball mill, weighed using a precision balance and encapsulated in tin for stable isotope analyses.

Isotope analyses were conducted at the Center for Stable Isotope Biogeochemistry, University of California-Berkeley (USA). The carbon isotope ratio of leaf material ($\delta^{13}C_L$) was analysed using elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Finnigan MAT Delta PlusXL IRMS). The oxygen isotope ratio of leaf material ($\delta^{18}O_L$) was determined with a Finnigan MAT Delta Plus XL IRMS (Finnigan MAT, Bremen, Germany) following the method described in Farquhar, Henry & Styles (1997) with some adaptations (Moreno-Gutiérrez et al. 2011). For $\delta^{18}O$ analyses of stem water, 0.2ml of water samples was equilibrated with an atmosphere of 0.2% of CO_2 for 48h at room temperature (21-23ºC). The $\delta^{18}O$ of equilibrated samples was measured using a continuous flow isotope ratio mass spectrometer (Finnigan MAT Delta Plus XL; ThermoFinnigan, Bremen, Germany) connected to a GasBench II interface (GB, ThermoFinnigan). δD of stem water samples was analysed using a chromium reduction system at 900ºC (Finnigan MAT H/Device) coupled to a Finnigan MAT Delta Plus XL mass spectrometer (ThermoFinnigan MAT, Bremen, Germany). Isotope ratios are expressed in delta notation (‰) relative to an accepted reference standard: V-PDB for $\delta^{13}C$ and V-SMOW for $\delta^{18}O$ and δD. Long-term (3+ year) external precisions for $\delta^{13}C$ and $\delta^{18}O$ of leaf material are 0.14 and 0.23‰, respectively. The long-term external precision for oxygen and hydrogen isotope analyses of water are 0.12‰ and 0.8‰, respectively.

Foliar phosphorus concentrations were measured at CEBAS-CSIC by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Elemental Iris Intrepid II XDL, Franklin, USA) after a microwave-assisted digestion with HNO_3:H_2O_2 (4:1, v:v). Foliar nitrogen concentrations were determined with a Thermo Finnigan Flash 1112 elemental analyzer (Franklin, MA, USA).

During each sampling campaign, plant height and crown diameter (measured at two directions) were recorded and plant biovolume was estimated as the volume of the upper-half ellipsoid with those height and diameters.

Statistical analyses

All statistical analyses were performed with SPSS software (version 17.0, SPSS Inc., Chicago). The responses of annual growth rings (from 1983 - when all plants were at least five year old - to 2007) and of $\delta^{13}C_R$ and $\delta^{18}O_R$ (from 1989 to 2007) were analyzed with a linear mixed-effect model, with stand type as the main effect (fixed factor), years as the variable to identify repeated observations and a first-order autoregressive covariance structure. The significance of the fixed effect term was assessed with Wald test and likelihood-ratio test. Individual trees were considered subjects. Simple Pearson correlations were used to examine the relationships between pairs of measured variables (meteorological variables and GRW_{res}, $\delta^{13}C_R$ and $\delta^{18}O_R$ chronologies) for each stand type separately. The relationships between analyzed variables and distance to *P. halepensis* were assessed with simple linear regressions.

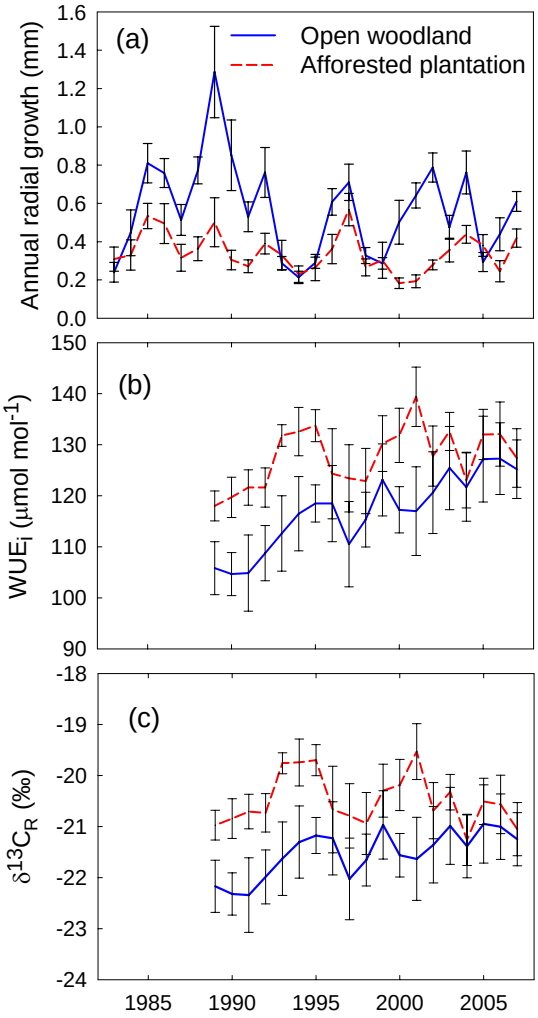

Figure 1. Open woodland and afforested stands mean chronologies of (a) annual radial growth of *R. lycioides* shrubs from 1983 to 2007 and (b) calculated intrinsic water use efficiency and (c) carbon isotopic composition of wood ($\delta^{13}C_R$) from 1989 to 2007. $\delta^{13}C_R$ values were corrected according to Francey *et al.* (1999) and McCarroll & Loader (2004). Error bars represent ± 1SE.

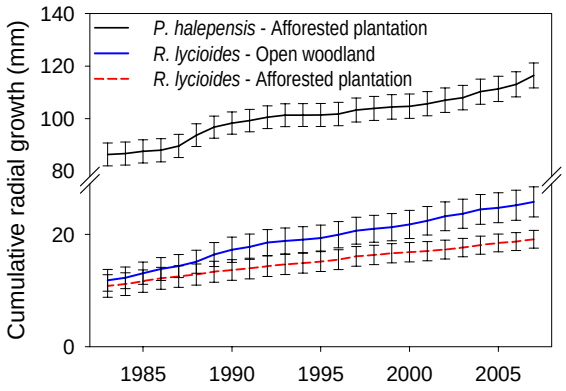

Figure 2. Average cumulative radial growth of *R. lycioides* shrubs in open woodlands and afforested stands and of *P. halepensis* in afforested stands (data from Moreno-Gutiérrez *et al.* 2012a). Error bars represent ± 1SE.

RESULTS

Radial growth analysis

There was not a significant relationship between the age of the *R. lycioides* shrubs and their mean growth ring width from 1983 to 2007, either within or across stand types. The dendrochronological characteristics of the residual mean chronologies standardized with ARSTAN are shown for each stand type in Table 1. The EPS value (*Expressed Population Signal*) of the open woodland chronology was very high (0.95), but the EPS value of the radial growth chronology from the afforested plantation was a bit lower (0.77; Table 1). However, the detrended radial growth chronologies from both stand types were significantly correlated with one another from 1983 to 2007 (r^2 = 0.20, P = 0.025), see Fig. 1a.

The growth rings of *R. lycioides* shrubs were significantly wider in the open woodland stands compared to the afforested stands from 1983 to 2007 (P < 0.001), and their mean values for that period were much greater in the open woodlands (0.56 ± 0.03 mm) than in the afforested stands (0.35 ± 0.03 mm). Therefore, stem width differences between stand types increased with time (see accumulated radial growth in Fig. 2), especially during favourable periods for growth when also *P. halepensis* trees showed high radial increments (see dotted vertical line in Fig. 2; radial growth data of pines was obtained from (Moreno-Gutiérrez *et al.* 2012a).

Growth-ring stable isotope composition

The intrinsic water use efficiency values (WUE_i), that were calculated from the carbon isotope composition of growth rings ($\delta^{13}C_R$),

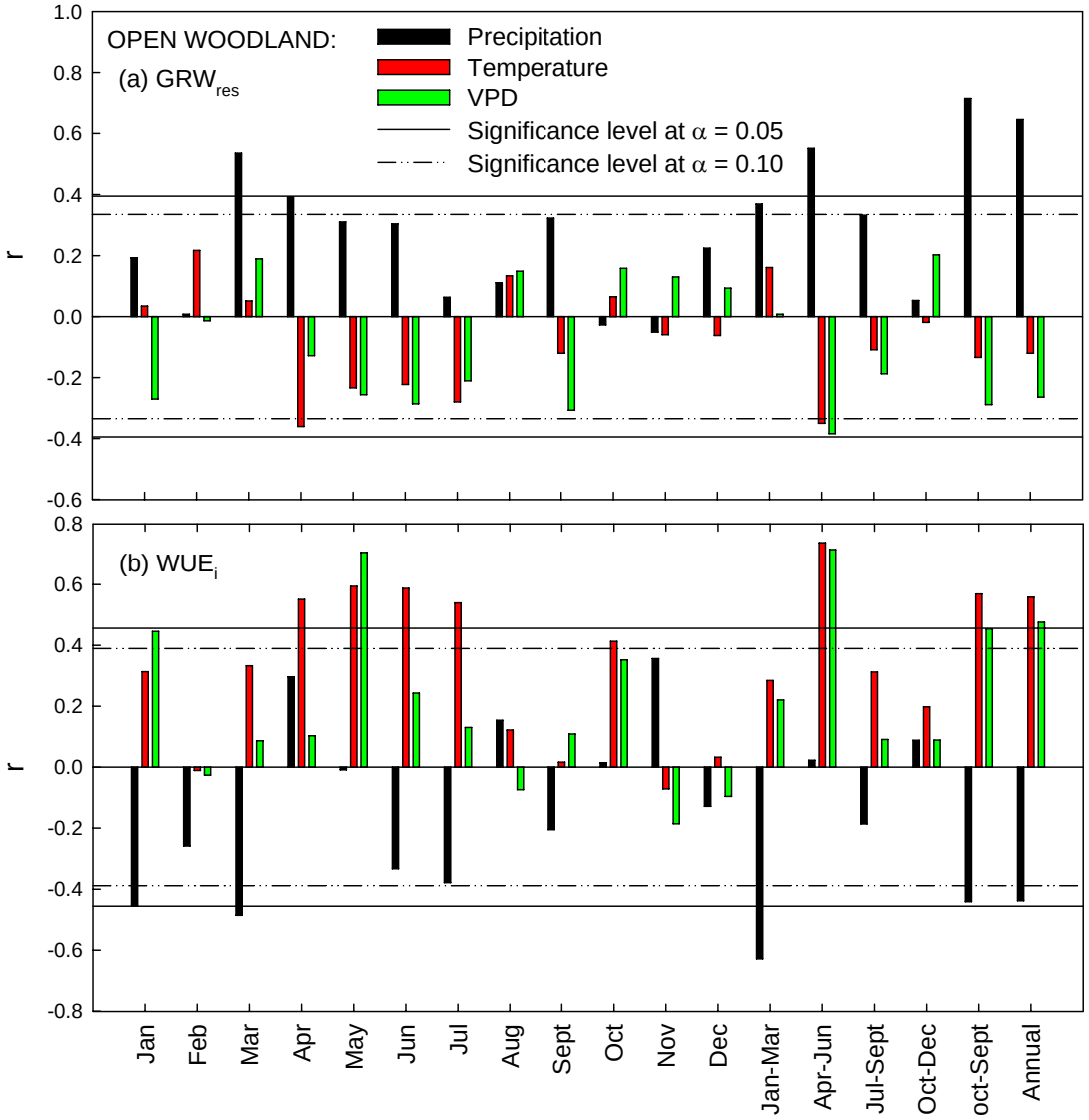

Figure 3. Pearson's correlation coefficients (r) and significance values (dotted lines) for the relationship in open woodlands between monthly/seasonal climatic variables and chronologies of (a) residual growth ring widths (GRW_{res}) and (b) intrinsic water use efficiency (WUE_i) calculated from wood carbon isotopic composition. "oct-Sept" represents the hydrological year, from October of the previous year to September of the current year.

were significantly higher in the afforested stands than in the open woodland stands for the period 1989 to 2007 (P = 0.026, Fig. 1b). Mean WUE_i values for that period were 126.7 ± 3.0 μmol mol^{-1} in the afforested stands and 116.6 ± 3.0 μmol mol^{-1} in the open woodland stands.

The WUE_i chronologies from both stand types showed similar fluctuations in the short-term (Fig. 1b) and were significantly correlated with one another from 1989 to 2007 (P = 0.005, r^2 = 0.38). However, the WUE_i values of shrubs in the open woodland stands followed an increasing trend with time (Fig. 1b), that was only observed in the afforested stands during the early years but disappeared afterwards. As a consequence, the difference between the WUE_i values from both stand types diminished with time (Fig. 1b).

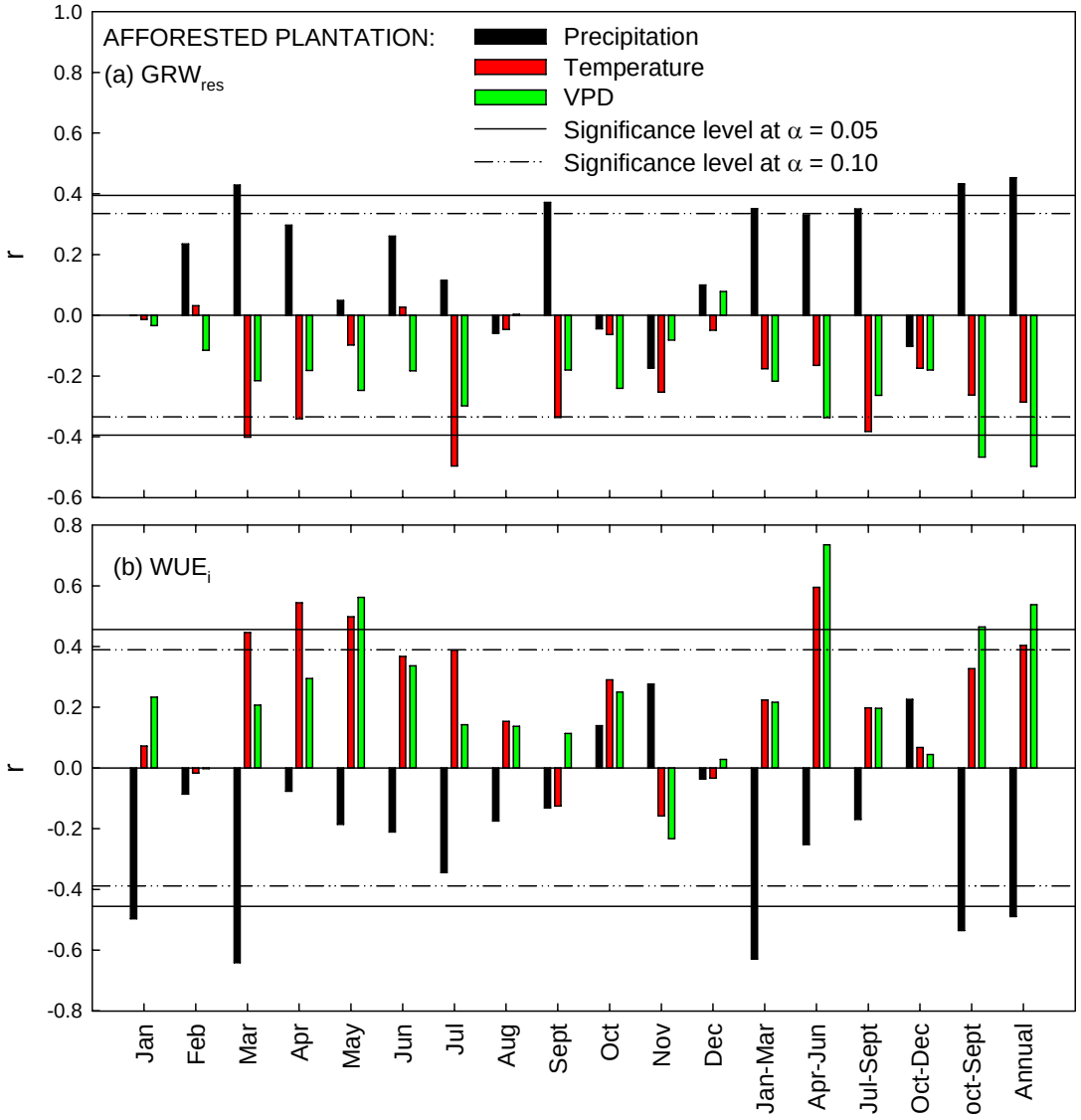

Figure 4. Pearson's correlation coefficients (r) and significance values (dotted lines) for the relationship in afforested stands between monthly/seasonal/annual climatic variables and chronologies of (a) residual growth ring widths (GRW_{res}) and (b) intrinsic water use efficiency (WUE_i) calculated from wood carbon isotopic composition. "oct-Sept" represents the hydrological year, from October of the previous year to September of the current year.

Interestingly, the WUE_i chronology was negatively correlated with the residual radial growth chronology in the afforested stands from 1989 to 2007 ($P = 0.019$, $r^2 = 0.28$), but this relationship was not significant in the open woodland stands. However, in the open woodland stands, residual radial growth was marginally significantly and negatively correlated with WUE_i when only years from 1989 to 1999 were considered ($P = 0.073$, $r^2 = 0.31$).

Relationships with climatic variables

The annual radial growth chronologies from 1983 to 2007 in both stand types were similarly correlated with climatic variables. Relationships with precipitation were of positive sign, whereas relationships with temperature and vapor pressure deficit (VPD) were of negative sign (Fig. 3a and 4a). However, correlations of radial growth with rainfall amount were stronger in the

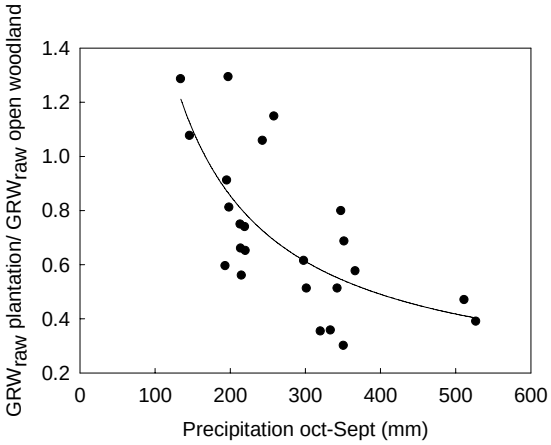

Figure 5. Inverse first order relationship between the ratio of mean annual radial growth of *R. lycioides* shrubs in afforested stands to open woodlands and precipitation from the hydrological year (from October of the previous year to September of the current year) when one outlier is not considered. $P < 0.001$, $r^2=0.50$, n=24.

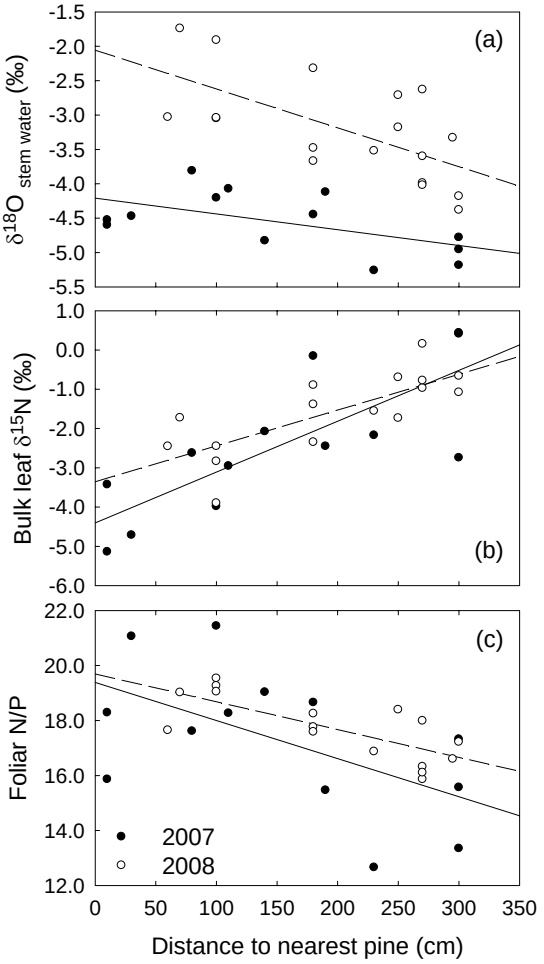

Figure 6. Relationship between the growing distance to the nearest pine of *R. lycioides* shrubs within afforested stands and their (a) stem water $\delta^{18}O$ ($P = 0.045$, $r^2 = 0.32$, n = 13 in 2007 and $P = 0.004$, $r^2 = 0.41$, n =18 in 2008), (b) bulk leaf $\delta^{15}N$ ($P = 0.002$, $r^2 = 0.61$, n =13 in 2007 and $P = 0.001$, $r^2 = 0.58$, n = 16 in 2008) and (c) ratio of nitrogen to phosphorous concentration of leaves ($P = 0.045$, $r^2 = 0.32$, n =13 in 2007 and $P = 0.001$, $r^2 = 0.55$, n = 16 in 2008) in years 2007 (closed symbols) and 2008 (open symbols).

open woodland stands than in afforested stands. In both stand types, radial growth was positively correlated with precipitation of the whole hydrological year (from October of the previous year to September of the current year, Poct-Sept, Fig. 3a and 4a) and precipitation of March. In the open woodland stands, growth was also strongly correlated with total precipitation from April to June (this relationship was only marginally significant in the afforested stands). On the contrary, temperature and annual vapor pressure deficit (VPD) exerted a stronger influence on the radial growth of shrubs in the afforested stands. Annual radial growth of shrubs was significantly correlated with temperature of March and July and with annual VPD in the afforested stands only (Figs. 3a and 4a).

The WUE_i of *R. lycioides* shrubs from 1983 to 2007 was negatively correlated with precipitation of the January to March period and of the whole hydrological year in both stand types (although the latter relationship was only marginally significant for the open woodland stands, Figs. 3b and 4b). In both stand types, WUE_i was positively correlated with VPD of May, VPD from April to June and with annual VPD (Figs. 3b and 4b). WUE_i was also positively correlated with mean temperature of the months of April and May and of the April to June period in both stand types. However, WUE_i was correlated with mean temperature of of June, July and the whole year in the open woodland stands only.

We calculated the ratio between annual radial growth of shrubs in the afforested stands and in the open woodland stands ($GRW_{plantation}/GRW_{open\ woodland}$) as indicator of relative growth differences between stand types. This ratio was negatively related to the total precipitation

amount of the hydrological year from 1983 to 2007 (polynomial inverse first-order regression; $r^2 = 0.50$, $P < 0.001$, when year 2000 was considered as an outlier; Fig. 5). During drought years the value of this ratio was close to 1, or even greater than 1, indicating that the radial growth of shrubs was similar in both stand types. Differences in radial growth between stand types became greater with increasing rainfall amount, and the ratio $GRW_{plantation}/GRW_{open\ woodland}$ decreased with precipitation.

Inter-species competition in dense afforested stands

The stem water $\delta^{18}O$ values of *R. lycioides* shrubs increased with decreasing distance to the nearest pine both in May 2007 and May 2008 (Fig. 6a; negative correlations between stem water $\delta^{18}O$ values and distance, $P = 0.045$, $r^2 = 0.32$, $n = 13$ and $P = 0.004$, $r^2 = 0.41$, $n = 18$, respectively). During a dry spring (2008) this relationship showed a steeper slope (Fig. 6a) than during a wetter spring (2007). There was a positive correlation between *R. lycioides* stem water content and distance to the nearest pine ($P = 0.006$, $r^2 = 0.52$, $n = 13$) in May 2007. Stem water content and stem water $\delta^{18}O$ values were in turn negatively related in May 2007 ($P = 0.034$, $r^2 = 0.35$, $n = 13$). However, these relationships were not significant during the dry spring of 2008, when all shrubs showed similarly low stem water contents (below 39%) regardless of their distance to the nearest pine.

The nutrient status of *R. lycioides* shrubs was also influenced by competition intensity by *P. halepensis* trees. The distance of *R. lycioides* shrubs to the nearest pine was strongly and positively correlated with leaf phosphorous concentration in May 2007 ($P = 0.002$, $r^2 = 0.61$, $n = 13$), and negatively correlated with the ratio between leaf nitrogen and phosphorous concentrations (N/P; $P = 0.045$, $r^2 = 0.32$, $n = 13$, in May 2007 and $P = 0.001$, $r^2 = 0.55$, $n = 16$, in May 2008; Fig. 6c).. When considering only shrubs growing at less than 200 cm from the nearest pine, leaf N concentration was also positively correlated with distance to the nearest pine in May 2007 ($P = 0.015$, $r^2 = 0.59$, $n = 9$). Distance to the nearest tree was positively correlated with leaf $\delta^{15}N$ composition ($P = 0.002$, $r^2 = 0.61$, $n = 13$, in May 2007 and $P = 0.001$, $r^2 = 0.58$, $n = 16$, in May 2008; Fig. 6b) We found positive correlations between leaf $\delta^{15}N$ and leaf P concentration ($P = 0.004$, $r^2 = 0.55$, $n = 13$ in May 2007 and $P = 0.079$, $r^2 = 0.20$, $n = 16$ in May 2008, marginally significant). In addition, leaf $\delta^{15}N$ composition was positively correlated with leaf nitrogen concentration in May 2007 ($P = 0.068$, $r^2 =$

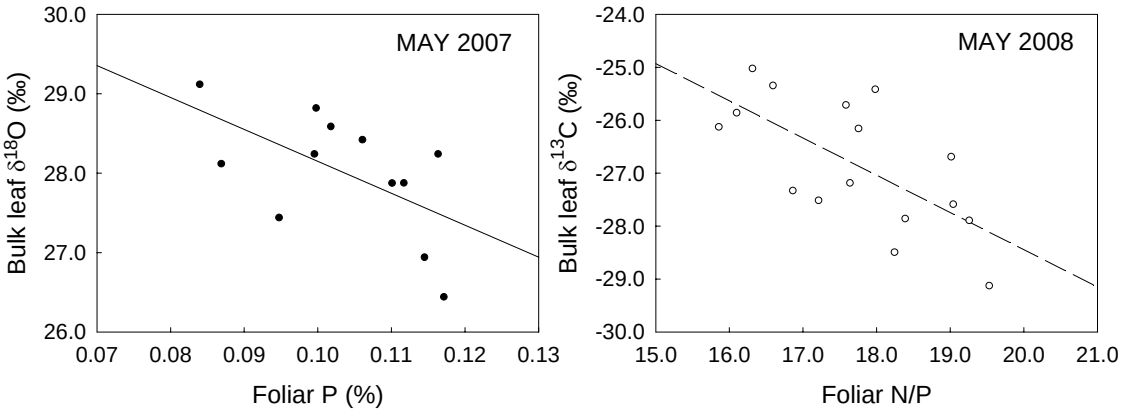

Figure 7. Relationships between (a) bulk leaf $\delta^{18}O$ and foliar phosphorus concentration in 2007 ($P = 0.047$, $r^2 = 0.34$, $n = 12$) and (b) bulk leaf $\delta^{13}C$ and the ratio of nitrogen to phosphorous concentration of leaves in 2008 ($P = 0.004$, $r^2 = 0.45$, $n = 16$) in *R. lycioides* shrubs growing within the afforested stands.

0.27, n = 13, marginally significant) and negatively correlated with leaf N/P in May 2008 (P = 0.032, r^2 = 0.31, n =15). Interestingly, stem water content was positively correlated with leaf $\delta^{15}N$ (P = 0.001, r^2 = 0.65, n =13), leaf phosphorous (P = 0.009, r^2 = 0.48, n =13) and nitrogen concentrations (P = 0.034, r^2 = 0.29, n =13) in May 2007.

The distance of *R. lycioides* shrubs to the nearest *P. halepensis* tree was marginally and negatively correlated with bulk leaf $\delta^{18}O$ (P = 0.067, r^2 = 0.30, n = 12) but not with leaf $\delta^{13}C$, in May 2007. Bulk leaf $\delta^{18}O$ was not significantly correlated with stem water $\delta^{18}O$ composition, but it was negatively related to leaf phosphorous concentration (Fig. 7a; P = 0.047, r^2 = 0.34, n = 12) and leaf N/P (marginally significant, P = 0.056, r^2 = 0.32, n = 12). In May 2008, the bulk leaf $\delta^{13}C$ and $\delta^{18}O$ of *R. lycioides* shrubs did not change with distance to the nearest pine. However, in May 2008, bulk leaf $\delta^{13}C$ composition was strongly related to plant nutrient status, which in turn was related to the distance to the nearest tree: bulk leaf $\delta^{13}C$ composition was positively correlated with bulk leaf $\delta^{15}N$ composition (P = 0.016, r^2 = 0.35, n = 16) and leaf phosphorous concentration (P = 0.011, r^2 = 0.34, n = 18) and was negatively correlated with leaf N/P (Fig. 7b; P = 0.004, r^2 = 0.45, n = 16).

Finally, the size (bio-volume) of *R. lycioides* adult shrubs was positively correlated with distance to the nearest *P. halepensis* tree in the afforested pine stands (P = 0.034, r^2 = 0.38, n = 12, in May 2007 and P = 0.018, r^2 = 0.30, n = 18, in May 2008).

DISCUSSION

R. lycioides long-term physiological performance and growth in *P. halepensis* plantations vs. open woodlands

R. lycioides shrubs growing in open woodlands showed wider growth rings than those in neighbouring afforested stands from 1983 to 2007 (Fig. 1a). In addition, *R. lycioides* shrubs in open woodlands showed lower intrinsic water use efficiency (WUE_i) than those in afforested stands from 1989 to 2007 (Fig. 1b). Greater radial growth and lower WUE_i in *R. lycioides* shrubs were related to increasing water availability, as these parameters showed negative and positive correlations with precipitation, respectively (Figs. 3 and 4). The higher WUE_i and lower radial growth of shrubs in dense afforested stands indicate that these shrubs had lower stomatal conductance, which limited their photosynthetic activity compared to shrubs in open woodlands. Similarly, Bota, Medrano & Flexas (2004) found that decreasing stomatal conductance had a dominant role in photosynthesis down-regulation during drought in two *Rhamnus* species (*R. alaternus* and *R. ludovici-salvatoris*). The higher density of pines in afforested stands translated into greater intensity of inter-specific competition for water and lower moisture availability for *R. lycioides* shrubs growing in afforested stands, thus leading to chronic low levels of stomatal conductance, photosynthetic activity and growth in these shrubs. Bellot *et al.* (2004) also found that increasing tree density in *P. halepensis* plantations reduced soil moisture availability for understory shrubs in a Mediterranean semiarid ecosystem. The fact that the differences in mean cumulative radial growth between stand types became significant during a period when pines in the afforested stands showed high radial growth increments (Fig.2) further supports the notion that afforestation with *P. halepensis* exerts a strong negative effect on *R. lycioides* performance.

Radial growth differences between stand types increased during rainy years (Fig.5), indicating that water availability was much greater for shrubs in open woodlands during wet periods. In contrast, shrubs in dense afforested stands were exposed to both canopy interception and intense inter-specific competition for soil water by neighbouring trees, which limited their growth during wet periods. During drought periods, the radial growth of *R. lycioides* shrubs was severely constrained by water stress in both stand types. In very dry years (annual rainfall < 200 mm), the ratio of plant radial growth in afforested stands to that in open woodlands

approached values around or above 1 (Fig. 5), indicating a possible facilitative effect of pines on *R. lycioides* performance. Closed forest canopies moderate temperature and maintain humidity in the forest understory, (Aussenac 2000) which may buffer *R. lyciodes* against climate extremes. Overall, these results support the stress gradient hypothesis (Bertness & Callaway 1994) that predicts increasing frequency of facilitative interactions under particularly stressful abiotic conditions.

The EPS value (*Expressed Population Signal*) indicates the level of coherence of the constructed chronology. Establishing an EPS threshold is subjective and depends on the particular goal of the study (Wigley, Briffa & Jones 1984), but a threshold of 0.85 is widely used to assess whether the constructed chronology is representative of the common growth signal of plants within the same community (Wigley *et al.* 1984). The EPS value of the radial growth chronology was very high in the open woodlands (0.95) but it was a bit lower in the afforested stands (0.77; Table 1). The intense inter-specific competition for water between *P. halepensis* and *R. lycioides* probably adds noise to the common climatic growth signal of *R. lycioides* shrubs in the afforested stands, thus leading to a lower EPS value. However, the common influence of climate on the radial growth of shrubs in the afforested stands was also evident, as growth was correlated with annual precipitation and VPD in the afforested stands (Fig. 4). In addition, chronologies from both stand types were positively correlated.

The WUE_i of *R. lycioides* shrubs in the open woodlands showed a long-term increasing trend. In the afforested stands, this increasing trend was observed until the late 1990s only. Other authors have also found similar increments in the intrinsic water use efficiency of trees during the last decades in response to increasing temperatures and atmospheric CO_2 concentration (Linares *et al.* 2009, Maseyk *et al.* 2011, Saurer, Siegwolf & Schweingruber 2004). Maseyk *et al.* (2011) found that this trend was particularly strong in *P. halepensis* trees growing in a semiarid ecosystem. Saurer *et al.* (2004) pointed out that this increasing trend in WUE_i corresponded to a scenario where plants adopted the strategy of maintaining constant internal CO_2 concentration inside the leaves (constant c_i). When atmospheric CO_2 concentration increases, plants can maintain constant c_i by decreasing stomatal conductance and increasing their intrinsic water use efficiency, without necessarily increasing their photosynthetic rate or growth. In agreement with this, Nock *et al.* (2011) found that the intrinsic water use efficiency of trees in a tropical monsoon forest increased with calendar year due to reduced stomatal conductance, and this was not translated into greater radial growth. In our study, the long-term increment in the intrinsic water use efficiency of *R. lycioides* shrubs was not accompanied by a similar trend in radial growth, further supporting the notion that decreasing stomatal conductance was mainly responsible of increasing WUE_i in response to higher atmospheric CO_2 concentration. This could also explain why changes in WUE_i appeared to be uncoupled from growth variations in *R. lycioides* shrubs growing in open woodland stands.

In a Mediterranean environment, Linares *et al.* (2009) found that *Abies pinsapo* trees have increased their WUE_i during the last decades. However this increment has been smaller in drier sites, where this trend reflects a scenario in which trees maintain constant c_i/c_a (ratio of internal to ambient CO_2 concentration), and therefore constant WUEi. This scenario seems to better describe the physiological performance of *R. lycioides* shrubs in afforested stands during the last years. As these shrubs suffered from stronger water stressed due to intense inter-specific competition for water by pines, they operated at lower stomatal conductance levels than shrubs in open woodlands. In afforested stands, climate aridification due to increasing temperatures could be critical and may have produced further reductions in stomatal conductance that could have induced a proportional down-regulation of photosynthetic activity. A strong stomatal limitation of photosynthetic activity in the shrubs of the afforested stands is further supported by the negative relationship

between WUE_i values and radial growth. In addition, the fact that the intrinsic water use efficiency of shrubs in afforested stands did not increase with time during the last 10-12 years could be related to other factors that could be co-limiting photosynthetic activity in these shrubs. For example, Tognetti, Cherubini & Innes (2000) pointed out that co-limitation by nutrients could counteract the beneficial effect that rising atmospheric CO_2 concentration can have on plant stem growth in dry sites.

Plant-plant interactions within *P. halepensis* plantations

R. lycioides shrubs growing in close vicinity to *P. halepensis* trees showed higher values of stem water $\delta^{18}O$ than those shrubs growing further away from trees (Fig. 6). The oxygen stable isotope composition of plant xylem water accurately reflects the isotope ratio of soil water used by plants, as no isotopic fractionation occurs during soil water uptake by roots (Barbour 2007). Soil water $\delta^{18}O$ can show steep vertical gradients during dry periods due to intense evaporation of water in soil surface and reduced evaporation effect with soil depth, thus allowing to differentiate among water sources used by plants in semiarid environments (Moreno-Gutiérrez *et al.* 2012b). Higher values of stem water $\delta^{18}O$ in *R. lycioides* shrubs growing in close vicinity to *P. halepensis* trees indicate that these shrubs use water from shallower soil layers than shrubs growing further away from pines. Moreno-Gutiérrez *et al.* (2012b) found that coexisting *R. lycioides* shrubs and *P. halepensis* trees exploit the same or very similar pools of water in this semiarid environment (i.e. they use water stored at same or similar soil depths). Our results suggest that there is a strong below-ground competition for soil water between both species, with *P. halepensis* trees out-competing *R. lycioides* shrubs and forcing them to rely on more superficial soil water, which is a highly fluctuating, and less abundant resource pool.

The negative effect that competition by *P. halepensis* exerted on the water relations of neighbouring *R. lycioides* shrubs was more evident during a wet spring (2007). During the drier spring of 2008, all shrubs in the afforested stands were similarly water stressed and showed similar low stem water contents regardless of the intensity of competition by *P. halepensis* trees. During the more mesic spring of 2007, *R. lycioides* shrubs growing in close vicinity to the nearest pine showed lower stem water contents and higher bulk leaf $\delta^{18}O$ values than those growing farther away, indicating reduced stomatal conductance in the former due to intense competition for water by *P. halepensis*. Bulk leaf $\delta^{13}C$ values did not change with distance to the nearest pine, indicating that reduced stomatal conductance caused a parallel reduction in photosynthetic activity and no change in intrinsic water use efficiency in *R. lycioides* shrubs growing at short distance from the nearest pine. Lower photosynthetic rates due to reduced stomatal conductance were finally reflected in bio-volume reductions in *R. lycioides* shrubs subjected to intense competition by *P. halepensis* trees.

Bulk leaf $\delta^{18}O$ values are known to be influenced by the $\delta^{18}O$ signal of the source water used by plants (Barbour 2007). However, bulk leaf $\delta^{18}O$ values were not related to the oxygen isotopic composition of stem water in *R. lycioides* shrubs, suggesting that interplant variations in bulk leaf $\delta^{18}O$ mainly reflect variations in the evaporative fractionation processes that take place in the leaves (and therefore, variations in stomatal conductance).

The competition intensity by *P. halepensis* trees also influenced the nutrient status of *R. lycioides* shrubs. Under higher levels of competition intensity (i.e., shorter distances from the nearest pine), *R. lycioides* shrubs showed lower leaf $\delta^{15}N$ values and higher leaf N/P ratios (Fig. 6). In May 2007 they also showed lower leaf nitrogen and phosphorous concentrations with decreasing distance from the nearest pine. The growth rate hypothesis states that rapidly growing organisms show low N/P ratios (Elser *et al.* 2010, Rivas-Ubach *et al.* 2012). Lower leaf N/P ratio with increasing distance to the nearest pine may thus reflect higher growth

rates of *R. lycioides* shrubs subjected to lower degree of inter-specific competition for resources by *P. halepensis*. In May 2008, bulk leaf $\delta^{13}C$ was strongly positively correlated to foliar nutrient concentrations and was negatively correlated with leaf N/P ratio, which was in turn influenced by competition intensity with pine trees. These relationships may reflect increasing photosynthetic activity and growth rate of plants with superior nutrient status in very dry years, when all shrubs were severely water stressed and changes in stomatal conductance with competition intensity were not evident. However, nutrient and water status were closely related in *R. lycioides* shrubs during a wet spring, as stem water content was positively correlated with leaf $\delta^{15}N$, leaf phosphorous and nitrogen concentrations in May 2007.

Leaf $\delta^{15}N$ values are often positively related to plant nutrient status in non-leguminous plants (Bai *et al.* 2009, Craine *et al.* 2009, Hobbie *et al.* 2000) and were indeed positively related to leaf nitrogen and phosphorous concentrations of *R. lycioides* shrubs in this semiarid environment. Increasing leaf $\delta^{15}N$ values with distance to the nearest pine could reveal utilization of different nitrogen sources by shrubs subjected to different degrees of inter-specific competition. Increasing soil moisture availability with distance to the nearest pine could be responsible for greater leaf $\delta^{15}N$ and nutrient concentration in *R. lycioides* shrubs, as at the local scale soil $\delta^{15}N$ is known to increase with moisture availability due to accelerated N-cycle processes (Bai *et al.* 2009, Handley *et al.* 1999). Variations in leaf $\delta^{15}N$ with distance from the nearest pine could also reflect drifts in the nitrogen source used by plants (e.g. nitrate or ammonium) due to changes in the competitive strength of plant-plant interactions (Kahmen, Wanek & Buchmann 2008) or changes in the soil depth at which plants extract nutrients, as soil $\delta^{15}N$ is known to increase with depth (Natelhoffer & Fry 1988), especially in sites dominated by ectomycorrhizal vegetation (Hobbie & Ouimette 2009) like in these afforested stands.

In conclusion, competition intensity with *P. halepensis* trees strongly influences the nutrient and water status of *R. lycioides* shrubs growing in afforested stands in this Mediterranean semiarid environment. Inter-specific competition for water was more evident during more mesic years, while all shrubs appeared to be severely water stressed in drier years irrespective of the intensity of competition with pines. In the long-term, shrubs subjected to strong inter-specific competition for water in dense afforested stands were not able to increase their intrinsic water use efficiency as an adaptive response to rising atmospheric CO_2 concentration. Climate warming and aridification produced parallel reductions of stomatal conductance and photosynthetic activity in *R. lycioides* shrubs from afforested stands, thus limiting their ability to respond and adapt to rising atmospheric CO_2. Finally, this study shows that stable isotope techniques are very useful to assess the short- and long-term outcome of inter-specific plant-plant interactions, and thus can help managers to select and adopt appropriate silvicultural practices that minimize the negative effects of competitive interactions among coexisting species in semiarid plant communities

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CAPÍTULO 6

DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

En ecosistemas áridos y semi-áridos el crecimiento de las plantas está fuertemente limitado por la disponibilidad de agua que es muy variable en el tiempo, se presenta en forma de pulsos y se distribuye de forma desigual a lo largo de los distintos horizontes del suelo (Schwinning & Ehleringer 2001). Esto condiciona los procesos ecológicos que determinan la organización de las comunidades vegetales. Se han descrito varios mecanismos que pueden explicar cómo las especies vegetales pueden coexistir en comunidades de ecosistemas áridos y semi-áridos (Chesson *et al.* 2004): las plantas pueden utilizar distintas fuentes de agua distribuidas a lo largo del perfil del suelo (como demostraron Williams & Ehleringer 2000) pero también pueden mostrar diferentes estrategias para usar los mismos pulsos de agua y aguantar los periodos secos (Goldberg & Novoplansky 1997) o también puede haber cierto grado de segregación temporal en la utilización del agua del suelo debido a desfases temporales en la fenología de las especies coexistentes (Werner & Máguas 2010).

En el ecosistema semiárido de pinar abierto con matorral que es objeto de estudio en la presente Tesis Doctoral, las 10 especies vegetales analizadas, pertenecientes a grupos funcionales muy distintos (esclerófilas perennifolias, deciduas o semi-deciduas durante la estación seca, una conífera y una herbácea), mostraron diferentes estrategias de utilización del agua (Capítulo 1). Estas especies presentaron diferencias en su eficiencia intrínseca en el uso del agua, formando un gradiente que va desde estrategias más conservadoras en el uso del agua hasta estrategias más derrochadoras (mayor cantidad de agua transpirada por C asimilado). Este gradiente está principalmente determinado por diferencias inter-específicas en el grado de control estomático de la fotosíntesis y de la transpiración a nivel foliar. En este ecosistema semiárido de tipo Mediterráneo, las especies de planta estudiadas mostraron marcadas diferencias en el grado de regulación estomática de la transpiración lo que resultó determinante a la hora de definir su estrategia de utilización del agua. Las distintas estrategias de las plantas y las diferencias inter-específicas en su conductancia estomática resultaron estar estrechamente ligadas a diferencias inter-específicas en la profundidad del suelo a la que las plantas extraen el agua. Las plantas que extraen agua a mayor profundidad mostraron las estrategias más conservadoras. Las estrategias más derrochadoras, con una mayor conductancia estomática, estuvieron asociadas a especies

de enraizamiento superficial, que dependen de una fuente de agua muy variable en el tiempo puesto que en los horizontes más superficiales del suelo el agua está sujeta a una fuerte evaporación directa y a una intensa competencia entre plantas por este recurso. La capacidad de estas especies para usar rápidamente los pulsos de agua y aprovecharlos al máximo durante los periodos en los que el agua está disponible en los horizontes superficiales del suelo parece estar relacionada con el carácter efímero de este recurso. Las especies de plantas deciduas o semi-deciduas mostraron las estrategias más derrochadoras, lo que refleja la existencia de una compensación entre la capacidad de las plantas para usar rápidamente los recursos durante los pulsos de agua y su habilidad para soportar lo períodos secos. Estas plantas evitan el estrés hídrico entrando en una dormancia vegetativa parcial durante la estación seca, que es típica de plantas con una estrategia elusiva o de escape frente al estrés hídrico (Valladares *et al.* 2004). Estos resultados validan en parte el modelo hidráulico de suelo-planta desarrollado por Schwinning & Ehleringer (2001), que predice que en ambientes dominados por pulsos de agua superficial se ven favorecidas las estrategias que maximizan el uso rápido de este recurso, para lo cual las plantas presentarían elevada conductancia estomática y enraizamiento superficial. En cambio, cuando la fuente de agua que predomina está en los horizontes profundos del suelo, ésta está disponible por más tiempo y es más constante, puesto que en estos horizontes del suelo los efectos de la evaporación directa y de la competencia por el recurso son menores. Cuando predomina esta fuente de agua, el modelo predice que se verán favorecidas las especies de enraizamiento profundo, que presentan estrategias más conservadoras y son capaces de extender su período de crecimiento durante parte del periodo seco.

En este ecosistema semiárido de tipo mediterráneo se combinan los paradigmas descritos en la bibliografía para ecosistemas áridos que explican la complementariedad del uso del agua de las diferentes especies coexistentes en base a su capacidad para explotar el agua disponible en diferentes horizontes del suelo (Ehleringer *et al.* 1991, Williams & Ehleringer 2000) y a sus distintas estrategias para aprovechar los pulsos de agua y soportar los periodos secos (Goldberg & Novoplansky 1997).

La complementariedad en el uso de los recursos hídricos resulta fundamental en ecosistemas áridos y semi-áridos para maximizar el uso del recurso más limitante para la vegetación y aumentar la productividad primaria total del ecosistema (O'Connor,

Haines & Snyman 2001). La diversidad de especies (y por tanto de estrategias) coexistentes favorece la multifuncionalidad de estos ecosistemas (Maestre *et al.* 2012). Mediante la segregación espacial y temporal en la utilización de las fuentes de humedad del suelo y el desarrollo de distintas estrategias de utilización del agua, las especies de plantas coexistentes se segregan en diferentes nichos ecohidrológicos, lo que disminuye el grado de competencia inter-específica por los recursos (Silvertown *et al.* 1999). Esto favorece la coexistencia de especies de plantas de tipos funcionales diferentes en las comunidades vegetales de ambientes áridos y semi-áridos.

Las relaciones de competencia y facilitación entre especies también son claves para determinar la composición y la estructura de las comunidades vegetales en ecosistemas áridos y semiáridos (Fowler 1986). La hipótesis del gradiente del estrés (stress gradient hypothesis) propone que la frecuencia de las interacciones de facilitación entre plantas aumenta en ecosistemas con un alto grado de estrés ambiental (Bertness & Callaway 1994). Sin embargo, esta hipótesis no se ha visto respaldada por algunos estudios llevados a cabo en ambientes semiáridos (Maestre *et al.* 2009), ya que la competencia entre plantas puede ser muy intensa en ecosistemas fuertemente limitados por el agua. En estos ecosistemas, el principal tipo de relaciones de competencia que se da entre plantas es la disputa por los recursos del suelo (Casper & Jackson 1997). En los últimos capítulos de la presente Tesis Doctoral hemos visto que las relaciones de competencia por el agua entre plantas de ecosistemas semiáridos pueden prevalecer en algunos casos, empeorando el estatus hídrico de las plantas y limitando su crecimiento.

El análisis de las interacciones entre plantas es especialmente importante en repoblaciones forestales de alta densidad, en donde la vegetación implantada puede verse forzada a competir fuertemente por los recursos del suelo. Las repoblaciones monoespecíficas de *Pinus halepensis* Mill. son muy frecuentes y ocupan amplias extensiones de territorio en el SE ibérico semiárido. Estas repoblaciones de carácter hidrológico-forestal se establecieron con el objetivo inicial de prevenir la erosión y la desertificación a corto-medio plazo, con la expectativa de que posteriormente serían colonizadas otras especies nativas propias de etapas sucesionales más avanzadas, de tal manera que a largo plazo evolucionarían hacia comunidades más naturalizadas (Cortina *et al.* 2011). Sin embargo, esto no ha sido así en muchos casos, y como resultado de esa

política encontramos en la actualidad masas forestales con una alta densidad de pinos que muestran escaso crecimiento (Maestre & Cortina 2004). Los Capítulos 2 y 3 ponen en evidencia el alto grado de competencia intra-específica por el agua que sufren los pinos dentro de repoblaciones de *P. halepensis* del SE ibérico semiárido. Al comparar los pinos de la repoblación con los que crecen dispersos en pinares abiertos con matorral, los primeros mostraron menor conductancia estomática, valores más bajos de potencial hídrico y menor crecimiento. El mayor grado de competencia intra-específica por el agua que hay en la repoblación empeora el estatus hídrico de los pinos. Esta especie es una especie isohídrica, evitadora del estrés que presenta un fuerte control estomático de la transpiración (Ferrio *et al.* 2003). Encontramos que los niveles de conductancia estomática y actividad fotosintética están fuertemente correlacionados en *Pinus halepensis*, indicando la importancia del control estomático en la difusión de CO_2 al interior de la hoja y por tanto, también en la actividad fotosintética. La alta densidad de pinos en la repoblación hace que aquí la transpiración a nivel de toda la comunidad sea mucho mayor que en la zona de matorral, lo que reduce las reservas de agua del suelo y afecta negativamente a la disponibilidad de agua individual para cada pino. Bellot *et al.* (2004) encontraron que al aumentar la densidad de pinos en repoblaciones de *P. halepensis* disminuía el contenido en humedad del suelo. En el ecosistema semiárido objeto de estudio en la presente Tesis Doctoral, la fuerte competencia intra-específica por el agua intensifica el estrés hídrico que sufren los pinos de la repoblación y disminuye su conductancia estomática de forma crónica (independientemente de las condiciones meteorológicas de cada año).

Sin embargo, estos pinos responden rápidamente a la disminución de la intensidad de la competencia por el agua. En tan sólo un año fueron evidentes los efectos positivos del aclareo de la masa forestal sobre los procesos fisiológicos a nivel foliar de los pinos. Los pinos que fueron sometidos a una mayor intensidad de aclareo (y por tanto a una mayor reducción del grado de competencia intra-específica por el agua) mostraron mayor conductancia estomática y actividad fotosintética. Los efectos sobre el crecimiento radial de los pinos tardaron más de un año en ser evidentes pero con el tiempo (2-3 años) los pinos de las parcelas más intensamente aclaradas crecieron más que los de las parcelas sometidas a un aclareo más moderado. En los capítulos 2 y 3 se pone de manifiesto la necesidad de actuaciones de manejo forestal como el aclareo

que disminuyan la intensidad de la competencia intra-específica por el agua, lo que podría mejorar el estatus hídrico de los pinos remanentes y mejorar la resiliencia de estas comunidades frente a la intensificación de la aridez asociada al cambio climático. McDowell *et al.* (2003) también demostraron la utilidad del aclareo para mejorar el estatus hídrico y el crecimiento de árboles remanentes de *Pinus ponderosa* en áreas fuertemente limitadas por el agua del suroeste de Norteamérica. La estructura de los pinares de repoblación podría mejorarse mediante la introducción de otras especies de plantas que presenten estrategias complementarias en el uso del agua respecto a la de *P. halepensis* y que por tanto maximicen la utilización de todas las fuentes de agua disponibles. Estas actuaciones podrían además acelerar los procesos de sucesión ecológica en estas masas forestales artificiales. Vilagrosa *et al.* (2005) destacaron el papel fundamental de la ecofisiología a la hora de aportar conocimiento para el diseño de repoblaciones forestales que simulen la estructura y funcionamiento de las masas forestales naturales. Los estudios de tipo ecofisiológico pueden complementar los enfoques más tradicionales basados principalmente en medidas de supervivencia y crecimiento, ya que aquéllos aportan información fundamental sobre los procesos fisiológicos de las plantas en respuesta a su medio.

En ecosistemas áridos y semiáridos las relaciones de competencia inter-específicas son muy importantes, aunque no sean tan frecuentes como en ambientes más mésicos, y determinan la estructura de las comunidades vegetales (Fowler 1986). Las relaciones de competencia también deben ser tenidas en cuenta a la hora de diseñar repoblaciones forestales. Maestre, Cortina & Bautista (2004) estudiaron un caso de competencia indirecta entre *P. halepensis* y *Pistacia lentiscus* (lentisco): la supervivencia de plantones de lentisco era mayor bajo la copa de los pinos que en los claros, indicando la ausencia de competencia directa por los recursos entre ambas especies. Sin embargo, la supervivencia de los lentiscos era aún mayor cuando se eliminaba el sustrato herbáceo, indicando de este modo la presencia de relaciones de competencia entre los plantones de lentisco y el sustrato herbáceo. Esto explicaría cómo los pinos, de forma indirecta, dificultan o impiden la colonización por especies leñosas nativas de su zona de influencia directa. Otras especies leñosas sí que compiten directamente con el pino carrasco por el agua del suelo, lo que puede hacer que estas especies no sean capaces de colonizar los pinares densos de repoblación (Bellot *et al.*

2004). En el último capítulo se presenta un caso de competencia inter-específica directa por el agua y los nutrientes entre *Rhamnus lyciodes* L. (espino negro) y *P. halepensis*.

En el primer Capítulo ya vimos que *R. lyciodes* y *P. halepensis* utilizan fuentes de agua similares, con parecida composición isotópica. En el último Capítulo se evidencia la fuerte competencia por los recursos del suelo entre ambas especies y cómo el pino prevalece en los pinares densos de repoblación, empeorando de este modo el estado hídrico de los espinos que crecen bajo su influencia. Estas relaciones son visibles a escala local dentro de los pinares de repoblación cuando se comparan espinos que crecen a diferente distancia del pino más próximo, y también a nivel de comunidad cuando se comparan espinos que crecen en comunidades vecinas de pinar abierto con matorral y pinares de repoblación. Los espinos que crecen dentro de la repoblación de pino carrasco mostraron mayor eficiencia intrínseca en el uso del agua y menor crecimiento radial (lo que implica que tienen también una menor conductancia estomática) que los espinos de pinares abiertos con matorral. Estas diferencias en el crecimiento fueron mayores en años más húmedos, anulándose o incluso invirtiéndose en años más secos. Esto concuerda con la hipótesis del gradiente del estrés (Bertness & Callaway 1994) que propone un aumento de las relaciones de competencia en ambientes más mésicos y un incremento de la facilitación cuando el estrés ambiental es mayor. De todas formas las relaciones de competencia entre especies prevalecen a largo plazo, de tal manera que los espinos de la repoblación mostraron mayor eficiencia intrínseca en el uso del agua durante el periodo de 19 años estudiado (aún teniendo en cuenta que estas diferencias han ido disminuyendo en los últimos años). El hecho de que los espinos de la repoblación tengan mayor eficiencia intrínseca en el uso del agua se debe a que soportan un mayor estrés hídrico crónico, lo que da lugar en una menor conductancia estomática debido a la fuerte competencia inter-específica por el agua con el pino carrasco. El análisis en detalle del estatus hídrico los espinos dentro de la repoblación muestra cómo los arbustos que crecen en proximidad al pino se ven forzados a utilizar fuentes de agua más superficiales, probablemente debido a que sus raíces son desplazadas por las raíces de los pinos de los horizontes más profundos del suelo donde la humedad es más constante. En una primavera húmeda, la intensa competencia interespecífica por el agua del suelo hace que los espinos que crecen en proximidad a los pinos muestren menor conductancia estomática (como indican sus valores más

enriquecidos de $\delta^{18}O$ foliar) y menor contenido en humedad de los tallos. Estas diferencias se atenúan durante la primavera más seca, cuando todos los espinos muestran un fuerte estrés hídrico, independientemente de su grado de proximidad a los pinos vecinos.

A lo largo de todos los Capítulos, las técnicas de isótopos estables se mostraron muy útiles para dar indicaciones de tipo ecofisiológico sobre los procesos que se dan en las plantas en relación con su medio y la disponibilidad de recursos en estos ecosistemas semiáridos del SE ibérico. Los valores de $\delta^{18}O$ del agua extraída de tejidos leñosos ayudaron a diferenciar las fuentes de agua que utilizan las plantas (Dawson *et al.* 2002) y resultaron útiles para determinar la profundidad del suelo a la que las distintas especies coexistentes extraen agua. Se trata de una información muy relevante, puesto que se vio que la profundidad de extracción de humedad del suelo condiciona la estrategia de uso del agua de las distintas especies vegetales en este ecosistema semiárido. Los valores de $\delta^{18}O$ y $\delta^{13}C$ foliar fueron buenos indicadores de las diferencias inter-específicas en la conductancia estomática y en la eficiencia intrínseca en el uso del agua, respectivamente. A pesar de que la composición isotópica del material vegetal puede verse afectada por fraccionamientos post-fotosintéticos o por diferencias inter-específicas en las características de las hojas (Barbour 2007, Offermann *et al.* 2011, Seibt *et al.* 2008, Warren & Adams 2006), la relación entre los valores de composición isotópica foliar y de intercambio de gases en hoja fue muy fuerte a nivel inter-específico. En este ecosistema semiárido fuertemente limitado por el agua las diferencias inter-específicas en los niveles de conductancia estomática se revelaron como el factor más importante que determinó la composición isotópica foliar y la estrategia de uso del agua de las distintas especies vegetales. Los valores de $\delta^{18}O$ y $\delta^{13}C$ de la hoja definieron un gradiente de estrategias de uso del agua entre especies pertenecientes a grupos funcionales diversos. Este gradiente se mantuvo entre años con distinta precipitación, indicando que la composición isotópica foliar de las distintas especies refleja propiedades inherentes de las plantas que se conservan a lo largo de los años. De este modo los valores de $\delta^{18}O$ y $\delta^{13}C$ foliar se revelaron como indicadores robustos de las estrategias de uso del agua de las plantas y por tanto se mostraron muy útiles como herramientas para evaluar la diversidad funcional de las comunidades vegetales en sistemas semiáridos. Arndt (2006) ya destacó la necesidad de investigar la

diversidad funcional de las comunidades vegetales para poder entender los procesos que determinan su organización y su respuesta a cambios ambientales.

Los valores de $\delta^{18}O$ de las hojas y de los anillos de crecimiento también resultaron ser buenos indicadores de la conductancia estomática a nivel intra-específico. Estos valores mostraron una buena correspondencia con los valores de conductancia estomática que se midieron por intercambio de gases en hoja en *P. halepensis* (Capítulos 2 y 3). Los pinos que estaban sometidos a distinto grado de competencia intra-específica por el agua mostraron diferencias en los niveles de conductancia estomática y esto se vio reflejado en los valores de la composición isotópica en oxígeno de las plantas. Algunos otros autores también han descrito una relación significativa entre los valores de $\delta^{18}O$ y el grado de conductancia estomática a nivel intra-específico (Barbour *et al.* 2000a, Barbour *et al.* 2000b, Grams *et al.* 2007).

Los valores de $\delta^{18}O$ foliar se mostraron especialmente útiles para evaluar a corto plazo el efecto del aclareo en la fisiología de los pinos remanentes. El $\delta^{18}O$ foliar respondió en tan sólo un año al efecto de la intensidad del aclareo, a diferencia de otras medidas convencionales como el crecimiento radial de los troncos, que tardó 2-3 años en responder al aclareo.

En este ecosistema semiárido, los valores de $\delta^{18}O$ de la celulosa de los anillos de crecimiento en *P. halepensis* preservaron claramente la señal del enriquecimiento isotópico de tipo evaporativo que se dan en el agua de la hoja (que depende de los niveles de conductancia estomática). Esto es relevante puesto que durante la formación de la celulosa en los anillos de crecimiento, una proporción importante de los átomos de oxígeno de la misma se intercambia con los de las moléculas de agua del xilema, de tal manera que los valores de $\delta^{18}O$ de la celulosa también reflejan la composición isotópica de la fuente de agua utilizada por la planta (Roden, Lin & Ehleringer 2000), con lo cual la señal del enriquecimiento evaporativo que se da en la hoja se atenúa durante la formación de la madera (Gessler *et al.* 2009, Jäggi *et al.* 2003). Ferrio & Voltas (2005) encontraron que los valores de $\delta^{18}O$ de los anillos de crecimiento de *P. halepensis* se correlacionan positivamente con el déficit de presión de vapor de agua cuando se comparan árboles de 23 localidades distribuidas en un gradiente de precipitación y temperatura representativo del rango de distribución de *P. halepensis* en la península ibérica. Sin embargo, los valores de $\delta^{18}O$ de los anillos de crecimiento no estaban

relacionados con los valores de $\delta^{18}O$ de la precipitación en estos pinos, indicando que en esta especie el enriquecimiento isotópico que se da en la hoja durante la transpiración prevalece sobre el efecto atenuante del intercambio de los átomos de oxígeno de la celulosa con el agua del xilema. En la presente Tesis Doctoral, los valores de $\delta^{18}O$ de los anillos de crecimiento de *P. halepensis* fueron distintos en dos tipos de comunidad vegetal con estructura muy diferente (pinares densos de repoblación vs. pinares abiertos con matorral), y se correspondieron con los valores de conductancia estomática medidos por intercambio de gases en hoja. En la repoblación forestal, los pinos mostraron valores de $\delta^{18}O$ más altos y niveles de conductancia estomática más bajos que los pinos que crecen en un pinar abierto con matorral, en donde la competencia intra-específica por el agua es mucho menor y por tanto la recarga de agua del suelo puede ser mayor.

Durante el periodo de 1967 a 2007, el $\delta^{18}O$ de los anillos de crecimiento de los pinos se correlacionó negativamente con la precipitación total del año hidrológico y positivamente con el déficit de presión de vapor de julio, indicando que estos valores responden a diferencias interanuales en la disponibilidad de agua que provocan variaciones anuales en los niveles de transpiración de los pinos. En cambio, los valores de $\delta^{18}O$ de los anillos de crecimiento no se correlacionaron con la temperatura media anual, lo que hubiera sido de esperar si la composición isotópica del agua de lluvia (fuertemente dependiente de la temperatura) hubiera sido la señal predominante en la celulosa de los anillos de crecimiento.

De forma similar, los valores de $\delta^{13}C$ de los anillos de crecimiento de *P. halepensis* se correlacionaron negativamente con la precipitación y positivamente con la temperatura y el déficit de presión de vapor. Esto indica que la eficiencia intrínseca en el uso del agua disminuye al aumentar la disponibilidad hídrica para los pinos debido a que éstos aumentan su conductancia estomática. Otros autores encontraron resultados similares en *P. halepensis* (Ferrio *et al.* 2003) y en *Fagus silvatica* (Saurer, Siegenthaler & Schweingruber 1995).

Sin embargo, a diferencia de los valores de $\delta^{18}O$, los valores de $\delta^{13}C$ de *P. halepensis* no variaron en respuesta a diferencias en el grado de competencia intra-específica por el agua: los valores de $\delta^{13}C$ de los anillos de crecimiento no fueron significativamente distintos en árboles de pinares con muy diferente estructura y grado de competencia intra-específica por el agua (Capítulo 2). Los valores de $\delta^{13}C$ foliar

tampoco mostraron diferencias significativas entre parcelas sometidas a diferente intensidad de aclareo (Capítulo 3). En casos como éstos en los que el $\delta^{13}C$ no varía pese a los cambios importantes en la actividad fisiológica de la planta, se hace aún más evidente la utilidad de la interpretación conjunta de los datos de $\delta^{13}C$ y $\delta^{18}O$ para discernir posibles cambios en la actividad fotosintética (A) y la conductancia estomática (g_s) (Grams *et al.* 2007, Scheidegger *et al.* 2000). En este ecosistema semiárido, los pinos sujetos a una menor competencia intra-específica por el agua (pinares abiertos con matorral o pinares de repoblación tras aclareo intenso) mostraron una mayor conductancia estomática, pero su ratio A/g_s no varió porque la actividad fotosintética también aumentó en paralelo, de tal manera que la eficiencia intrínseca en el uso del agua se mantuvo constante y por tanto no hubo cambios en los valores de $\delta^{13}C$. Probablemente, una mayor disponibilidad de nutrientes y de luz también contribuyó a que la actividad fotosintética fuera mayor en los pinares con menor densidad de arbolado (Warren, McGrath & Adams 2001). En el caso del $\delta^{13}C$ de los anillos de crecimiento también hay que considerar que los procesos de fraccionamiento post-fotosintético pueden haber atenuado la señal isotópica original del carbono a nivel de hoja (Gessler *et al.* 2009, Offermann *et al.* 2011) haciendo de este modo que el $\delta^{13}C$ de los anillos de crecimiento no registre las pequeñas diferencias en eficiencia intrínseca en el uso del agua que puedan darse entre comunidades vegetales.

A diferencia de *P. halepensis*, en *R. lycioides* los valores de $\delta^{13}C$ de los anillos de crecimiento sí mostraron diferencias significativas entre comunidades sometidas a distinta intensidad de competencia inter-específica por el agua. En ambientes estacionalmente secos, en donde la evaporación es mayor que la precipitación anual, los valores de $\delta^{13}C$ pueden ser buenos indicadores de la disponibilidad de agua para las plantas (Hartman & Danin 2010, Stewart *et al.* 1995, Warren *et al.* 2001). En el caso de *R. lycioides*, los valores de $\delta^{13}C$ de los anillos de crecimiento reflejaron fuertes diferencias en la disponibilidad hídrica entre los dos tipos de pinar: los espinos que crecen en el pinar abierto con matorral mostraron mayor crecimiento y menor $\delta^{13}C$ (menor eficiencia intrínseca en el uso del agua) que los espinos sometidos a una fuerte competencia inter-específica por el agua en la repoblación de *P. halepensis*. Todo ello indica que los espinos del pinar abierto con matorral tienen un ratio A/g_s más bajo (pese a tener una mayor actividad fotosintética) debido a que tienen una conductancia

estomática mucho mayor gracias a que la disponibilidad hídrica aquí es mayor que en la repoblación.

En conclusión, las técnicas isotópicas proporcionan una herramienta muy potente en estudios de ecofisiología vegetal en ecosistemas limitados por el agua. En la presente Tesis Doctoral se muestran claros ejemplos de la utilidad de las técnicas de isótopos estables para entender los procesos fisiológicos que se dan en las plantas en respuesta a diversos factores ecológicos bióticos y abióticos en ecosistemas semiáridos. La medición de la composición isotópica del material vegetal resulta particularmente útil para entender las relaciones hídricas de las plantas y el resultado del balance entre el consumo de agua y la adquisición de carbono.

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CAPÍTULO 7

CONCLUSIONES GENERALES

CONCLUSIONES

Las conclusiones más importantes de la presente Tesis Doctoral son:

1. En este ecosistema semiárido de tipo mediterráneo, las 10 especies vegetales más representativas muestran estrategias de utilización del agua diferentes entre sí y potencialmente complementarias, lo que puede contribuir a maximizar el aprovechamiento del agua a nivel de comunidad vegetal. Las diversas estrategias están determinadas principalmente por diferencias inter-específicas en los niveles de conductancia estomática y en la profundidad de extracción del agua en el perfil edáfico, y determinan un gradiente que va desde estrategias más "derrochadoras" (elevada conductancia estomática, g_s; baja eficiencia intrínseca en el uso del agua, WUE_i; y uso de agua del suelo superficial) hasta las más "conservadoras" (baja g_s; elevada WUE_i; uso de agua almacenada en los horizontes más profundos del suelo).

2. Los valores de $\delta^{18}O$ y $\delta^{13}C$ foliar proporcionan robustos indicadores de las diferencias inter-específicas en la conductancia estomática y en la eficiencia intrínseca en el uso del agua, respectivamente. Al realizar comparaciones inter-específicas, es recomendable analizar la $\delta^{18}O$ de la celulosa, puesto que ésta resultó ser un mejor indicador de la conductancia estomática que la composición isotópica en oxígeno del material foliar en bruto.

3. A nivel inter-específico, los valores de $\delta^{18}O$ y $\delta^{13}C$ foliar reflejaron un gradiente de estrategias de uso del agua que se mantuvo en años con distintos niveles de precipitación, indicando que las especies de plantas muestran "nichos isotópicos" característicos que pueden resultan útiles para evaluar la diversidad funcional de las comunidades vegetales en ecosistemas semiáridos.

4. En las repoblaciones forestales con alta densidad de plantación de pino carrasco (*P. halepensis)* se producen intensas relaciones de competencia por el agua entre los pinos, lo que afecta negativamente al estatus hídrico del arbolado y disminuye su potencial hídrico, su conductancia estomática y su actividad fotosintética.

5. En este ecosistema semiárido, el grosor de los anillos de crecimiento de *P. halepensis* y sus valores de $\delta^{18}O$ y $\delta^{13}C$ responden fuertemente a las variaciones interanuales en la precipitación (periodo 1967-2007). Además, la composición isotópica en C y O de la celulosa de la madera se correlaciona negativamente con el crecimiento radial de los pinos. En este ecosistema fuertemente limitado por el agua, las variaciones inter-anuales en $\delta^{18}O$ de los anillos de crecimiento de *P. halepensis* reflejan principalmente el enriquecimiento isotópico de tipo evaporativo que se da a nivel foliar, proporcionando una medida integrada en el tiempo de los niveles de conductancia estomática y transpiración de los árboles.

6. El aclareo de las masas forestales repobladas de *P. halepensis* (pino carrasco) mejora significativamente el estatus hídrico de los pinos remanentes. A mayor intensidad de aclareo se logra una mayor reducción de la competencia intra-específica por el agua y aumenta la conductancia estomática, la actividad fotosintética y el crecimiento radial de los pinos remanentes. El aumento de la actividad fotosintética en los pinos remanentes de las parcelas más intensamente aclaradas se debe a una menor limitación estomática a la difusión del CO_2 atmosférico al interior de la hoja.

7. Las medidas de de $\delta^{18}O$ foliar se mostraron especialmente útiles para evaluar a corto plazo el efecto del aclareo en la ecofisiología de los pinos remanentes. Transcurrido un año desde la aplicación del aclareo, el $\delta^{18}O$ foliar de los pinos remanentes reflejó fielmente el incremento de la conductancia estomática provocado por el aclareo.

8. Los valores de $\delta^{18}O$ foliar fueron menores en las parcelas más intensamente aclaradas, indicando niveles mayores de conductancia estomática en los pinos remanentes de estas parcelas. Sin embargo, los valores de $\delta^{13}C$ foliar de los pinos remanentes no cambiaron significativamente con la intensidad de aclareo. La interpretación conjunta de los datos de $\delta^{18}O$ y $\delta^{13}C$ foliar indicó que tanto la conductancia estomática como la actividad fotosintética de los pinos remanentes fueron significativamente mayores en las parcelas intensamente aclaradas que en aquellas donde se produjo un aclareo de intensidad moderada. Esta interpretación

de los datos isotópicos foliares se validó mediante medidas de intercambio de gases en hoja y medidas de crecimiento radial de los pinos. .La interpretación conjunta de los datos de $\delta^{13}C$ y $\delta^{18}O$ foliar resultó particularmente útil para discernir los efectos de los cambios en la actividad fotosintética y de la conductancia estomática en la composición isotópica en carbono del material foliar.

9. En las repoblaciones de *P. halepensis* también se desarrolla una fuerte competencia por los recursos del suelo (agua y nutrientes) entre la especie arbustiva *R. lyciodes* (espino negro) y *P. halepensis*. El pino compite de manera más eficiente que el espino por el agua almacenada en los horizontes más profundos del suelo, y fuerza al espino a utilizar la humedad de los horizontes más superficiales donde la evaporación y la competencia son más intensas. Los estatus hídrico y nutricional de *R. lycioides* están fuertemente interrelacionados y los espinos que crecen más cerca de los pinos también mostraron menores concentraciones foliares de nitrógeno y fósforo (y mayor N/P ratio, característico de plantas con menores tasas de crecimiento). De este modo, una mayor proximidad a ejemplares de *P. halepensis* afecta negativamente al estatus hídrico y nutricional de los espinos en pinares densos de repoblación de ecosistemas semiáridos.

10. Los espinos que crecen en pinares densos de repoblación sufren una mayor competencia inter-específica por los recursos del suelo que los espinos que crecen en pinares abiertos con matorral. Los anillos de crecimiento de éstos últimos fueron más gruesos y mostraron valores más bajos de $\delta^{13}C$ (desde 1989 a 2007), lo cual indica una mayor actividad fotosintética debido a una menor limitación estomática. Sin embargo, el efecto de la competencia inter-específica por el agua se hace menos evidente en años especialmente secos, en los que tanto los pinos como los espinos sufren un fuerte estrés hídrico. En años muy secos, el crecimiento radial de los espinos es incluso mayor en los pinares de repoblación que en los pinares abiertos, lo cual sugiere un posible efecto facilitador por parte de los pinos cuando las condiciones son especialmente estresantes.

11. Las técnicas de isótopos estables se mostraron muy útiles en este ecosistema semiárido mediterráneo para entender los procesos ecofisiológicos que se dan en las plantas. Son especialmente útiles para estudiar las relaciones hídricas de las plantas y el balance agua-carbono de la planta en respuesta a factores ambientales y al efecto de las interacciones con otras plantas. A lo largo de la presente Tesis Doctoral las técnicas de isótopos estables ayudaron a caracterizar las estrategias de uso del agua de especies muy diversas que coexistenten en este ecosistema semiárido. También ayudaron a detectar y evaluar fenómenos de competencia y facilitación relacionados con el agua a nivel intra- e inter-específico y a evaluar los efectos de tratamientos selvícolas sobre el estatus ecofisiológico del arbolado. La medición de la composición isotópica de los anillos de crecimiento permite ampliar el rango temporal que abarcan los estudios ecofisiológicos y es útil para evaluar los efectos de la variabilidad climática interanual sobre el estado ecofisiológico de la vegetación leñosa.

CONCLUSIONS

The main conclusions of this PhD Thesis are:

1. In this Mediterranean semiarid ecosystem, 10 of the most representative plant species showed contrasting water use strategies. These strategies can be complementary and may thus foster plant species coexistence and maximize the amount of water used by plants at the community level. Inter-specific variations in the water use patterns of plants were largely determined by differences in stomatal conductance and the depth of soil water extracted by plants. Plant species were ordered along a continuous ecophysiological gradient of water use strategies ranging from "profligate/opportunistic" (high stomatal conductance, g_s, and low intrinsic water use efficiency, WUE_i) to "conservative" (low g_s, high WUE_i).

2. Across plant species, leaf $\delta^{18}O$ and $\delta^{13}C$ were reliable indicators of stomatal conductance and intrinsic water use efficiency, respectively. When performing comparisons across species, it is more appropriate to analyze the oxygen stable isotope composition of leaf cellulose, as it was more strongly correlated with stomatal conductance than the oxygen isotope composition of bulk leaf material.

3. The ten co-occurring species segregated along a continuous ecophysiological gradient defined by their leaf $\delta^{13}C$ and $\Delta^{18}O$ and maintained their relative isotopic rankings in two years with contrasting rainfall, which suggests the existence of species-specific "isotopic niches" that reflect ecophysiological niche segregation in dryland plant communities

4. Within dense afforested plantations of *P. halepensis*, intense inter-tree competition for water is developed, thus deteriorating the water status of pines and diminishing their water potential, stomatal conductance and photosynthetic rates.

5. In this semiarid ecosystem, the ring width and isotope composition ($\delta^{13}C$ and $\delta^{18}O$) of *P. halepensis* trees were strongly correlated with rainfall amount (and thus, with soil water availability) at inter-annual scales (from 1967 to 2007). In addition, the carbon and oxygen isotopic composition of tree ring cellulose was

negatively correlated with radial growth of pines. In this strongly water-limited ecosystem, inter-annual variations in the $\delta^{18}O$ of *P. halepensis* tree rings are mainly related to the evaporative isotopic enrichment that takes place in the leaves, thus providing an integrated measure of pines' stomatal conductance and transpiration.

6. Thinning of *P. halepensis* afforested plantations improves significantly the water status of the remaining trees. Heavy thinning reduces inter-tree competition for water more effectively than moderate thinning, thus resulting in higher stomatal conductance, photosynthetic activity and radial growth in the remaining trees. The greater photosynthetic activity of the remaining trees in the heavily thinned stands is due to a lower stomatal limitation to the diffusion of atmospheric CO_2 inside the leaves.

7. Measuring leaf $\delta^{18}O$ was especially useful to asses the ecophysiological response of trees to thinning in the short term. One year after thinning, the bulk leaf $\delta^{18}O$ of the remaining trees reflected the increment of stomatal conductance produced by thinning.

8. Remaining trees in low-density stands (heavily thinned) showed lower $\delta^{18}O$ and higher stomatal conductance than those in moderate-density stands (moderately thinned). By contrast, leaf $\delta^{13}C$ was unaffected by stand density. The dual isotope interpretation of bulk leaf $\delta^{13}C$ and $\delta^{18}O$ indicated that the remaining trees in heavily thinned stands showed higher stomatal conductance and photosynthetic rates than pines in moderately thinned stands. This interpretation of isotopic data was in good agreement with leaf gas exchange data and stem radial growth of pines. The dual isotope approach was especially useful in this semiarid ecosystem to distinguish between the effects of changes in stomatal conductance or photosynthetic rates on the carbon isotope composition of leaf material.

9. Within *P. halepensis* afforested plantations, there is also a strong inter-specific competition for water and nutrients between *P. halepensis* and *R. lyciodies*. *P. halepensis* trees out-compete *R. lycioides* shrubs and force them to rely on more superficial soil water, which is a highly fluctuating and less abundant resource

pool. Nutrient and water status of *R. lycioides* shrubs are strongly inter-correlated and *R. lycioides* shrubs growing in close vicinity to the nearest pine showed lower concentrations of leaf nitrogen and phosphorous (and greater N/P ratio, characteristic of plants with lower growth rates). The water and nutrient status of *R. lycioides* shrubs are negatively affected by competition intensity with *P. halepensis* trees in dense pine afforested plantations of semiarid ecosystems.

10. *R. lycioides* shrubs growing within *P. halepensis* afforested plantations suffer stronger inter-specific competition for soil resources compared to *R. lycioides* shrubs growing in open woodlands. *R. lycioides* shrubs growing in the afforested plantation showed higher growth ring $\delta^{13}C$ values and lower radial growth than those in open woodlands, which indicates lower stomatal conductance and impaired water relations in the former. The intense competition for soil resources between *P. halepensis* and *R. lycioides* was less evident during dry years when all the shrubs were severely water stressed. In very dry years, radial growth of *R. lycioides* shrubs was even greater in the afforested stand than in open woodlands, thus indicating a possible facilitative effect of pines when environmental conditions are especially stressful.

11. Stable isotope methodologies were very useful in this semiarid ecosystem to understand plant ecophysiological processes. They are especially useful to asses the water dynamics of plants and their water-carbon balance in response to environmental factors and interactions with other plants. Along the current PhD Thesis, stable isotope techniques helped to characterize the water use strategies of coexisting plant species representing diverse taxonomic groups and life-forms. They also helped to identify intra- and inter-specific relationships of competition and facilitation and to assess the effect of silvicultural practices on the ecophysiological status of trees. Measuring the stable isotope composition of growth rings allows expanding the temporal range of ecophysiological studies and is useful to assess the effect of inter-annual climate variability on the physiological status of woody plants.

CONTRIBUCIONES CIENTÍFICAS

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Contribuciones a congresos:

Moreno-Gutiérrez C., Battipaglia G., Cherubini P., Saurer M., Siegwolf R., Nicolás E., Querejeta J.I. The long-term vulnerability of *Pinus halepensis* trees to drought stress is strongly modulated by stand structure in a semiarid Mediterranean ecosystem. 12th EEF Congress, septiembre 2011. Ávila (España). [Comunicación oral]

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